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The Editor comments

DILUTING SCIENCE WITH POLITICS

We have become greatly disturbed in recent months by the increasing influence being exerted by political factors in what properly should be classified as strictly scientific considerations.

American ability in the field of science is recognized the world over, and no small part of the nation's more able and dedicated scientific personnel are affiliated with government agencies such as the Public Health Service, Food and Drug Administration, Department of Agriculture, National Bureau of Standards, and various others. Traditionally, the activities of these dedicated people have been of a noncontroversial nature and in fact have been the object of little, if any, fanfare. On the contrary, in the past, many commentators both outside and within government have been moved to decry that all too little notice or recognition has been accorded these scientists, their useful work, and often brilliant accomplishments.

Lately, however, a number of the more clever political tacticians have found that government scientific activities can provide a very fertile field from which they may derive much publicity, thereby drawing national attention and eventually swaying public opinion. Several such incidents, including the classic case of the 1962 drug amendments, attest to the success of maneuvers of this nature.

As noted above, this influence is most disturbing to us, because among other things, we regard such a climate as extremely unhealthy for the proper growth and further development of true science. The trend of the present environment is becoming less conducive to objective, unbiased decision-making on the part of our government scientists, and lends itself to unfortunate compromises on their part in drawing conclusions and taking actions which *should* be based entirely upon an accurate evaluation of the facts involved.

If the present movement is not soon checked, not only may our nation suffer from the immediate effects resulting from emotionally engendered legislation and enforcement, but even more seriously, it appears that the entire tenor of true objectivity within our scientific community might well be in jeopardy.

Edward G. Feldmann

JOURNAL OF **Pharmaceutical Sciences**

January 1963 volume 52, number 1

Review Article

Plastics in Pharmaceutical Practice and Related Fields. Part I

By JOHN AUTIAN

CONTENTS

[Contents for Part II will accompany the concluding part of Dr. Autian's Review Article next month.]

INTRODUCTION

No material has received so much attention, found so many useful applications, and presented so many challenges as a group of chemical agents making up a body of dissimilar substances collectively grouped under the generic term, plastics. No longer a laboratory curiosity of several decades ago, plastics have become an everyday way of life in our complex society. No item or product from the simple dish stand in the kitchen to the complexly engineered space capsules has escaped the touch of one or more plastic materials. The ingenuity of the polymer chemist and the skill of the product engineer make it possible for the plastic industry and all its related affiliates to create and produce products which to a previous generation either were made from the more conventional materials or could not be made at all. Plastics have had a profound effect upon our economy and no doubt will become an economic necessity in the future if our nation is to maintain its accustomed high standard of living.

Even though the use of plastic materials had reached large proportions in other areas, its introduction to pharmacy and medicine was of more recent times and in more limited applications. Natural reluctance by the various health professions and by those servicing these professions to introduce new, untried materials without careful analysis for potential harm prevented the same degree of expansion as seen in many other fields. This state of affairs, however, has changed and is continuing to change as may be witnessed by reviewing the number of items used in pharmacy and medicine made in part or whole of a plastic substance. The many advantages which can be fashioned from the proper plastic material will undoubtedly give further impetus to the use of plastics in the health professions.

The rapid introduction of plastic items, such as containers, syringes, tubings, sheetings, prosthetic devices both internal and external, and a host of other items, to the practice of pharmacy and medicine has not been without certain difficulties. It appears that a greater scientific body of knowledge must be accumulated to guide the manufacturer to produce a plastic item which will repeatedly behave in an identical manner under various conditions of storage and use and which will insure that no possible harm will directly or indirectly fall upon the patient. In recent years there has been some concern generated on the possible harmful effects on animals and humans in the use of plastics in medicine. No attempt will be made here to review the toxicity aspect of plastics. The reader if interested in this facet of the subject is encouraged to ferret out some of the references listed (1–8).

As the outline indicates, the review will cover a number of topics which the author felt would be pertinent to the overall presentation of plastics to a pharmaceutical group. Some topics have received more attention than others but this should not suggest that these other topics are of minor importance for it certainly is recognized that to certain individuals and groups these less emphasized topics may indeed be of paramount importance. The reader may also be distressed in not seeing certain facets on the subject of plastics covered at all in this review. Here the author has taken some liberty in the selection of the material in order to conserve space and to limit the discussion to those areas which would seem to be appropriate to pharmacy and medicine. No one review can cover adequately any subject and this is even more true of a subject which has so many applications and which is in a continual state of growth. It is hoped, however, that what will be presented here will serve as a source of useful information to all those interested in finding more and better uses of plastics while at the same time alerting others to the problems which may be created by the use of plastics if proper precautions are not taken.

History

The impetus to our present "world of plastics" can be directly and logically traced back to the years of the Second World War, when a sudden need developed in both the Allied and Axis camps to find and produce in sufficient volume suitable replacements for scarce war materials. Scientific and engineering talents met the challenge by the expanding use of well-established plastics as well as exploiting those polymeric materials which were hardly more than laboratory curiosities. It was natural that this pool of knowledge, personnel, and equipment would be directed to civilian needs after the war years and to the present. One must, however, go much further back in history than the Second World War to assign a time for the invention of the first plastic.

A lack of adequate supply in the 1860's of ivory for the production of billiard balls inspired a printer by the name of John Wesley Hyatt to experiment with pyroxylin. In 1868 he fashioned an ivorylike material which soon became known as Celluloid, a combination of pyroxylin and camphor (9). The new material became the

Received from the Drug-Plastic Research Laboratory, College of Pharmacy, University of Texas, Austin 12.

Editor's note: Additional considerations of plastics in pharmaceutical practice will be discussed in the concluding portion of this review, Part II, which will appear in the February issue of This Journal.

first commercial plastic and soon was being used as denture plates, parts of wearing apparel (wipe-clean collars, cuffs, etc.), windows, photographic film and in 1882, the first motion picture film.[1] Fraenkel (10) in 1890 recorded the first published information in the use of Celluloid in surgery as a replacement of bone in a portion of the skull of a human.

Celluloid reigned supreme as the only plastic material for a period of four decades until Dr. Leo Henrik Baekeland in the year 1909 developed a phenol-formaldehyde resin which was given the name of *Bakelite* for its creator (9). The '20's and '30's saw the introduction of other plastic materials which, in a real sense, gave rise to a new industry—the plastic industry. As a brief review of the chronological history of plastics development, the reader should see Table I.

TABLE I.—A CHRONOLOGICAL HISTORY OF PLASTICS DEVELOPMENT[a]

Date	Material
1868	Cellulose nitrate
1909	Phenol-formaldehyde
1909	Cold molded
1919	Casein
1926	Alkyds
1926	Aniline-formaldehyde
1927	Cellulose acetate
1928	Polyvinyl chloride
1929	Urea-formaldehyde
1935	Ethyl cellulose
1936	Acrylic
1936	Polyvinyl acetate
1938	Cellulose acetate butyrate
1938	Polystyrene or styrene
1938	Nylon
1939	Polyvinyl acetals
1939	Melamine-formaldehyde
1939	Polyvinylidiene chloride
1942	Polyester
1942	Polyethylene
1943	Silicones
1943	Fluorocarbons
1945	Cellulose propionate
1947	Epoxy
1948	Acrylonitrile-butadiene-styrene
1956	Acetal resin
1957	Polypropylene
1957	Polycarbonate resin
1959	Chlorinated polyether

[a] "Modern Plastics Encyclopedia"—Issue for 1962, New York, p. 18.

Each of the plastics has an interesting story and one must go to published texts on plastics to appreciate the scientific minds of individuals and groups who helped synthesize these materials. Swallow (11) gives a very revealing and at times amusing account of the developments leading up to the first isolation of polyethylene, which as the author points out really commenced in 1932 when he (Swallow) and Perrin, working for Alkali Division of I.C.I., England, recommended that studies on chemical reactions at high pressures should be conducted. Now that polyethylene was prepared, what possible market would be open to it? On this point Swallow writes:

> Much is written, today, on the way in which a new product should be developed with a view to shortening the time between its discovery and its large scale production and commercial availability. To use phraseology of today it would have been difficult at that time to have carried out a "Market Survey" which would have led to any profitable conclusion, but here again the role of chance played a part.

The chance Swallow refers to was the interest by those in the submarine cable field to find a material which would replace the insulation used for submarine telephone cables and in 1939 enough material was produced to insulate one nautical mile of submarine cable. The entry of England into the World War in the same year provided the coming of age for polyethylene and perhaps the saving of England during the early years of conflict. English scientists were working feverishly to develop a super-secret detection device (radar) for announcing the coming of enemy planes over their island. It became clear to the scientists that if radar was to be a practical success a new material must be found for insulating cables and other components of radar which could effectively handle high voltages needed in radar detection. The new material, it was soon to be found, was polyethylene. Perhaps no greater tribute could be paid to this plastic than by the inventor of radar, Sir Robert Watson Watt, who said: "And so polyethylene played an indispensable part in the long series of victories in the air, on the sea, and on land, which were made possible by radar" (12).

In the field of plastics one must recognize the contribution of many individuals from many countries but several deserve added attention, as for example W. H. Carothers (U. S. A.—nylon), Dr. K. Ziegler (Germany—low pressure method for producing plastics), and Professor Natta (Italy—isotactic plastics). It appears that the real history of plastics is yet to come. What has been recorded for the accomplishment of plastics in the past will be of small magnitude to what is to be expected in the future of the material called plastics.

Definitions

At the onset, one point must be made clear to the reader. Plastics cannot and should not be considered as one type of material but rather

[1] A point of reference should be made here. Polystyrene has been known for over a hundred years and, in fact, a German pharmacist, Simon, is credited with the discovery of the styrene monomer in 1839 but its commercial use had to wait until around 1938.

an unlimited number of substances. By far the most appropriate definition thus far given may be stated as follows (13):

> A large and varied group of materials which consist of, or contain as an essential ingredient, a substance of high molecular weight which, while solid in the finished state, at some state in its manufacture is soft enough to be formed into various shapes, usually through the application, either singly or together, of heat and pressure.

Plastics may further be divided into two general classes: (*a*) the *thermosets*, those substances which when heated to a melt and cooled will not regain their original identity, and (*b*) *thermoplastics*, those substances which will regain their original identity. There are instances where a plastic material can be made to behave as either one or the other but for the most part one may consider a plastic as either belonging to the one class or the other.

Because of the unusual versatility of thermoplastics, they have found a wider application than the thermosets especially in the medico-pharmaceutical field. For this reason the main emphasis will be placed on this group of plastics in the present review.

Often the term *resin* is used in plastics technology and indicates the polymer or polymers making up the plastic material. A number of plastics can be prepared for specific applications without the addition of any other ingredient to the resin but others may contain beside the resin, plasticizers, antioxidants, stabilizing agents, fillers, antistatic agents, colorants, or other specific agents, to impart a definite quality to the final plastic product. These other ingredients may vary in concentration from a few parts per million parts of resin up to as high as 60% of the total weight of the plastic.

In recent years new dimensions have been added to plastics by chemically combining different monomers to form polymers. These polymers can then be considered as being composed of two types of plastics. The term *copolymer* is used to distinguish these plastics from those which have been synthesized from one type of monomer.

Once the basic resin is manufactured in bulk quantities, one or more steps of processing are usually necessary to have the material in suitable form for actual use in making a specific item. Depending upon the specific plastic item, other steps may be necessary in fabricating and finishing the item before it is actually ready for market. Clearly, it is not a simple chain of events. Much scientific technology and art come into play with many groups adding their particular touch to the final product. It may thus be appreciated why there can be so many variations in a plastic product even though for all intents and purposes the product is designated with a specific generic name such as polyethylene, polypropylene, etc.

GENERAL INFORMATION ON A SELECTED NUMBER OF PLASTICS

To gain the most from plastics, one must have working knowledge of the various plastic materials which are currently being used in the medico-pharmaceutical field and, further, one must be cognizant that no one material can be selected which will give all the advantages without any disadvantages for a particular application. Each application is a special project and must be treated as such. The more information which is available to the user of the plastics, the less time and effort will be required in selecting the proper material or at least in setting a scheme which will permit proper evaluation of the plastic for the specific application. There is no doubt that the packaging department of a pharmaceutical firm must be highly concerned with such matters.

The following section will include a discussion of a number of plastics which are currently being used. It will be obvious that not all materials will be dealt with and that rather brief accounts will be made of the ones discussed. The variety of materials which could be discussed under the term plastics would be too staggering for one paper and probably would not serve to the best interest of the reader. Those plastics which have been included are, however, representative of materials which have or probably will have application to the pharmaceutical field and to the related medical sciences. Certain omissions and simplifications have also been necessary in order not to weight the contents of the discussion.

Polyethylene (14, 15, 16)

Polyethylene, as the name clearly indicates, is a long chain polymer prepared from ethylene. Depending upon the mode of synthesis and the presence of selective catalyst a variety of polyethylenes can be prepared. It is, however, possible to classify polyethylenes into three general classes by simply considering the density of the polymeric material. These classes are: (*a*) *low density* (may also be called conventional, regular, branched, or high pressure), (*b*) *medium density*, and (*c*) *high density* (may also be called linear, low pressure). The low density is the original polyethylene which is prepared at

extremely high pressures (15,000 to 30,000 p.s.i.) while high density can be synthesized at moderate pressures with suitable catalysts. Medium density polyethylene may be prepared by a modification of the high-pressure method or by combining portions of low and high-density polyethylene.

Little information can be revealed on the structure of polyethylene by simple chemical methods other than elucidating the empirical structure, $(CH_2)_n$ etc. Various physical methods of analysis have been used and are being used to describe the structure. It is known, for example, that the polymer molecule may have from 800 to 100,000 carbon atoms, primarily in a straight chain structure. In the early days of polyethylene it was believed that each molecule had one terminal ethylene group and one or two methyl groups in each molecule. With improvement in instrumental analysis, it was soon realized that the original model of the polymer (low density) was in error and that the polymer contained branched chains, a number of methyl groups (approximately two or three per 100 CH_2 groups), several types of unsaturations and several polar sites in the form of carbonyl or keto groups. A composite of these structures may be seen in Fig. 1 taken from Kresser (17).

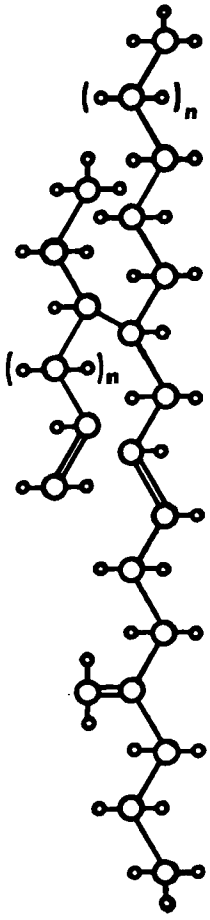


Fig. 1.—Polyethylene molecule showing some characteristic structures. Large circles represent carbon atoms; small circles, hydrogen atoms; double lines represent double bonds. [From Kressler, T. O. J., "Polyethylene," Reinhold Publishing Corp., New York, N. Y., 1961, p. 41.]

As with most polymers, the size of the polymer will play a very important role in imparting certain physical and mechanical properties to a finished plastic material. Unlike pure chemical compounds, polyethylene as well as other plastics, is not composed of an exact molecular size but rather that a distribution of molecular sizes are present. It is thus impossible to assign an exact molecular weight to a particular polyethylene and whatever value is assigned is some form of an average value. The determination of even these average molecular weights for high polymers is not a simple task and consequently highly refined physicochemical methods are used in an attempt to find a molecular weight. Since there are great technical problems associated with molecular weight determinations of high polymers, a number of methods for expressing molecular weight or size are evident in current practice and one must understand there may be considerable differences of values from one method to another. Techniques for these determinations are found in standard texts on plastics or polymers (18, 19).

One general method of expressing molecular weight of an unfractionated sample of polyethylene is to use the number-average molecular weight ($\bar{M}_n$) which may be depicted as

$$\bar{M}_n = \frac{\Sigma M_i n_i}{n_i} \qquad \text{(Eq. 1)}$$

where M_i is the molecular weight of molecules of a definite size i, and n_i, the number of that size. Measurement of colligative properties such as boiling point elevation, osmotic pressure, and freezing point depression of polymeric solutions will lead to the number average-molecular weight ($\bar{M}_n$).

A more convenient, but indirect, method for approximating $\bar{M}_n$ is through viscosity measurements. A number of equations have been developed connecting viscosity with molecular weight but in general they may be reduced to the empirical relation

$$\eta_0 = K\bar{M}_n^a \qquad \text{(Eq. 2)}$$

where η_0 is the intrinsic viscosity, K a constant,

and $\bar{M}_n^a$ the number-average molecular weight raised to a power, a. Often, rather than using molecular weight values, viscosities are used to convey molecular size and it can thus be understood why often comparison of data from one polyethylene to another becomes quite impractical.

Another expression of molecular weight, referred to as the weight-average molecular weight ($\bar{M}_w$) can be determined from light scattering experiments. This method actually measures a mean radius of a molecule in solution and not the molecular weight since two molecules having the same molecular weight may show different results if one has more branching in the chain than the other. The ratio $\bar{M}_w/\bar{M}_n$, however, helps to reveal the distribution of molecular weights in the same polymer, the larger the ratio, the broader the distribution.

Since it was early shown that polymers could be characterized by rheological properties, a standardized technique was devised to measure the viscosity of a polymer under specified conditions. The term *melt index* (*MI*) came into being and was defined as the number of grams of molten polymer which will flow through a standard orifice at a standard temperature and pressure (20). Unfortunately this method of measurement was soon found not to be very meaningful except for control purposes since two polymers produced by different manufactures but having the same melt index did not necessarily behave in the same manner during processing. The melt index, however, may be used as crude indication of molecular weight in an inverse fashion, *i.e.*, the higher the melt index the lower the molecular weight.

The various methods used to depict the size of polymers, as already stressed, is a form of an average. It would be extremely helpful if more information could be obtained on the exact distribution of the molecular sizes in a given polymer but technical difficulties which as yet have not been sufficiently overcome prevent reasonably accurate size frequency distributions to be charted. Attempts have been made, however, to fractionate polyethylene and thereby to reveal a molecular distribution with some limited success. In general the distribution has been found to be extremely broad. Important advantages may be gained by keeping the molecular weight distribution in a narrow range. For example, it has been found that such properties as tensile strength, stress cracking resistance, film impact strength, and low brittleness can be improved if the molecular weight distribution is narrowed.

It has now been clearly established that polyethylene as well as other plastics have in essence two zones of structure running through the material: a crystalline zone and an amorphous zone (see Fig. 2). Crystallization occurs when the polymer chains orient themselves side by side to form a more compact configuration. In a given polyethylene sample these compact zones of polymers, or crystallites, are dispersed throughout the amorphous zone of the material. The ratio of crystalline to amorphous structures in polyethylene impart various physical properties to the polyethylene. For example, the density of the crystalline zone by X-ray measurements indicates a density of 1.00, while for the amorphous zone, a density of 0.76 to 0.85 has been found. Conventional polyethylene has approximately 60 per cent of crystalline structure while high density polyethylene has a much greater content of crystallites.

Bunn (21) has made intensive studies on the crystalline zones of polyethylene and has found that the unit cell has an orthorhombic structure with $a = 7.40$Å, $b = 4.93$Å, and $c = 2.53$Å. The crystallites are not separate entities as may be found in metals but rather the chains composing the crystallites run through one or more amorphous zones as may be shown in the highly diagrammatic model in Fig. 2.

Thin samples of polyethylene show clarity while thicker samples display an opaque appearance. The opacity is due to the scattering of light which was thought to be due to the individual crystallites in the sample, but it is now known that the phenomenon of opacity is due to larger structural units than a crystallite. These larger units form a spherulite which comes about by proper arrangement of a number of small crystallites forming a nearly distinctive zone radiating outward from a central point (22).

A number of methods for evaluating the crystal structure of polymers have been devised (23, 24). Perhaps the most useful techniques are X-ray diffraction, infrared, density, nuclear magnetic resonance, and electron microscopy studies. It should be apparent that a number of factors will alter the ratio of crystalline to amorphous composition of a plastic material. Some of these factors are (*a*) structure of the molecule *i.e.*, chain length, degree of branching, length of chains in the various branches, degree of unsaturation, presence of other constituents, etc., (*b*) the temperatures, and (*c*) mechanical treatment (drawing or pulling the plastic material). An example of the effect of temperature on polyethylene may be noted by observing

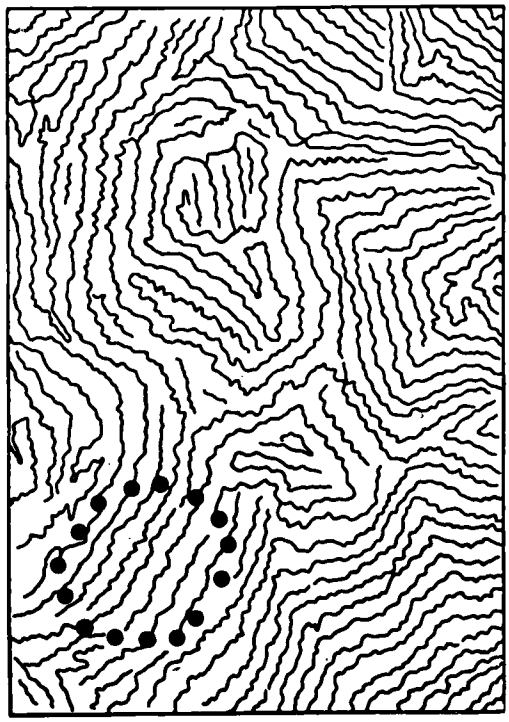


Fig. 2.—Plane projection of chains showing amorphous and crystalline (enclosed in dots) regions. [From Kinney, G. F., "Engineering Properties and Applications," John Wiley & Sons, Inc., New York, N. Y., 1957, p. 11.]

Table II where the degree of crystallinity is shown to decrease with an increase in temperature.

Mechanical treatment of a plastic sample such as pulling the material along one axis will help orient the polymers by straightening out the chains so that they become more parallel and closer to each other. This treatment favors crystalline structures in the plastic.

The degree of crystallinity in polyethylene will be a determinant for certain applications. For example, by experience it has been found that as the crystallinity of the material increases, stiffness increases, suface hardness increases, chemical resistance increases, permeability decreases, and film toughness decreases.

From what has been said it should be clear that the selection of a particular polyethylene for a specific application requires much care and it is wise not to succumb to the use of one or two parameters in defining the quality of the polyethylene. A prudent user should define his particular polyethylene by a number of parameters including important data such as density, melt index, molecular weight distribution, chain structure, and branching. The user should also have a complete background of the history of the plastic with pertinent and detailed information on additives which have been added directly or which have been introduced in the processing of the material.

The list of applications for polyethylene is in a sense unlimited. Production in 1962 amounted to 1.45 billion pounds (25). In medical and pharmaceutical practice much use is being made of this material for containers both as bottles and as film packaging materials, tubings, syringes, and other units for the administration of drugs and blood or for the collection of body fluids. Polyethylene coating on various surfaces has ushered in many new uses which could not be anticipated a few years ago.

Polypropylene (26)

In 1954 Professor Natta of the Institute di Chimica Industriale del Politenico, Milan, Italy, introduced a new plastic material which was synthesized by stereospecific polymerization. This new material was polypropylene which in a period of a few years has created a mild sensation for product designers because of certain advantages over the polyethylene line. Perhaps even more important than the introduction of a new plastic material was the sudden intense interest of polymer scientists in a new field of stereopolymers which may make possible newer plastic materials having superior properties over the conventional materials.

Commercial use of polypropylene started in 1958 and by 1961 had reached a production of approximately 80 million pounds which is estimated to increase to 425 million pounds by 1965 (25).

Polypropylene is colorless and odorless with a density of 0.90–0.91, which makes this material one of the lightest plastics known. The physical properties of polypropylene are in direct relationship to the molecular structure and to the geometry of the constituents in the chain.

TABLE II.—EFFECT OF TEMPERATURE ON THE CRYSTALLINITY OF POLYETHYLENE[a]

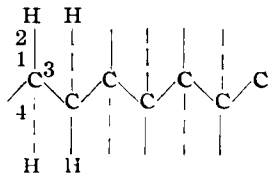
Temperature, °C.	Wt., % Crystalline Material
0	55
20	55
40	55
50	55
60	55
70	55
80	50
90	45
95	45
100	40
105	35
110	25
115	10

[a] Hunter, E., and Oakes, W. G., *Trans. Faraday Soc.*, **41**, 49 (1945).

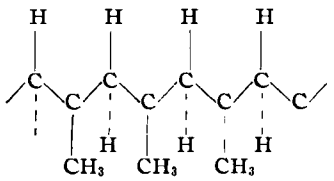
Two types of polypropylene are possible. The first of these will be called an *atactic* polymer while the second an *isotactic* polymer. First, however, it will be necessary to define or at least to visualize another term which will be designated as *tacticity*. If a polymer is composed of molecules whose basic units follow each other in the same spatial configuration, a tactic polymer is achieved. Depending upon the particular polymer, many possibilities arise for a variety of tacticities, even though only two will be discussed in this section.

The definition of atactic thus becomes a simple matter and indicates that no tacticity is present (no steric order present). Isotactic may be defined as a polymer in which parts of the molecule are oriented in space in the same manner for each unit of the polymer.

It will be helpful in understanding the term isotactic to compare the polymerization of ethylene and propylene to produce polyethylene and polypropylene, respectively. In the polymerization of ethylene, one molecule will join another by rupture of the double bonds forming, in an idealized fashion, a chain which may be depicted as



where lines 1 and 3 are in the plane of the paper, line 2 is from the paper toward the reader, and line 4 (dotted line) passes from the paper downward at the same angle as line 2. The structure depicted above is perfectly symmetrical and cannot be isotactic. Propylene on polymerization will follow the same addition steps to become a structure which might be visualized as



Here it will be noted that an unsymmetrical structure results with every other carbon being bonded to a methyl group. Since each methyl group is in the same spatial position (coming up from the paper) this molecule is isotactic. If the methyl groups were distributed in a random fashion (*i.e.*, some up and some down), the polymer would be referred to as an atactic polymer.

Another term now must be introduced, *syndiotactic* polymer. This refers to a structure whereby the methyl group alternates up and down in a regular pattern.

The above structure for polypropylene is for convenience and it should not be interpreted that in reality the chain would exist in this fashion. In fact it would be extremely difficult for several of these chains to approach each other since the methyl groups from two chains would repel each other. In an isotactic polypropylene, the chain forms a spiral, one spiral in three propylene units. The spiral formation permits the interlocking of two or more polymers into tight segments which in turn give rise to the crystal structure in polypropylene.

It is possible to prepare 100% isotactic polypropylene even though most commercial samples are never this high. In general, as the isotacticity increases, crystallinity increases in approximately the same order and often this relationship holds fairly well but it must be emphasized that there is no numercial correlation between the two. As with polyethylene, other factors will influence crystallinity, particularly time-heat treatment. This fact should caution one to be alert in designating polypropylene by only one or the other parameter (crystallinity and tacticity) if a specialized use is to be made of the polypropylene.

The degree of crystallinity, the size and shape of the individual crystallites, in any plastic material will affect both the physical and mechanical properties of the material. For example, it has been found that small and uniform crystallites will cause less stress points in the material. Crystallite size in polypropylene is approximately 150Å in diameter and 50–60Å thick. These crystallites form larger aggregates (spherulites) and it is necessary to control the growth of these spherulites since large sizes will invariably weaken points in the plastic which in turn lead to cracks or breaks (26).

Usual methods of molecular weight determination are used to characterize polypropylene but these values may be in gross error or may not really serve a useful purpose. Viscosity measurements appear to be the most used method of correlating molecular weight and it appears that such a factor as molecular weight distribution is an important consideration in designing a specific use for the plastic. Greater difficulties, however, are encountered in fractionating polypropylene than polyethylene in an attempt to collect size distribution data and newer techniques must be developed before reliable information can be accumulated.

As may be anticipated with a molecule containing a hydrogen attached to a tertiary carbon, polypropylene is more susceptible to oxidation than the polyethylenes and the inclusion of an antioxidant is a necessity for sustaining product life. Polypropylene is quite resistant to chemical attacks and products prepared from this plastic can be repeatedly autoclaved without any real harmful effect. Presently the material is being used for films, bottles, jars, household items, and industrial machinery to mention a few uses. In medicine and pharmacy the material is finding use as bottles, jars, syringes, cups, basins, sanitary ware, tubings, laboratory ware, and film packaging material. In completing this section, it is imperative to stress that varied properties, sometimes quite different, can exist between two or more polypropylenes. One should not surmise, therefore, the failing or success of a particular polypropylene product to mean that exact results will be duplicated when another polypropylene is used.

Polyvinyl Chloride (27, 28, 29)

One of the most versatile plastics is polyvinyl chloride (*PVC*) produced from the monomer vinyl chloride in a number of ways. Its present importance may be recognized by noting that approximately 950 million pounds of the homologous polymer and its copolymers were manufactured in this country in 1961 (25).

The simplest structure representing *PVC* may be shown with alternating (up and down) atoms of chlorine throughout the chain. If the chain is considered in its most extended form, repeating units occur at a distance of 5Å along the chain. Since the structure reveals asymmetric carbon atoms, isotactic polymers are conceivable but as yet practical realization of this type of material with *PVC* has not occurred. Viscosity measurements as well as other physicochemical studies reveal little indication that branching occurs in the polymer. Commercial samples of *PVC* vary in molecular weight from around 50,000 to 150,000 and most of these samples show a wide distribution of molecular weights.

Unmodified *PVC* is a hard, transparent plastic. In general, 60% of the material is in an amorphous state. On long standing in the presence of light, *PVC* shows a tendency to decompose by releasing hydrogen chloride. This reaction is accelerated with temperature and catalyzed by traces of zinc and iron. It is, therefore, necessary to include from one to two per cent of a suitable stabilizer to ensure an acceptable material. All types of *PVC* are susceptible to chemical attacks with organic materials such as ketones, esters, aromatic, and chlorinated hydrocarbons.

Polyvinyl chloride has unique properties which permit it to be modified in numerous ways giving a spectrum of end uses not seen with the other plastic materials. By far the most important modifying agent is the plasticizer. Often this ingredient may constitute up to 60% of the total weight of the finished material.

The use of plasticizers is not new, even though their economic impact is of more recent times. Early recorded history reveals that oil was added to pitch in order to make it pliable enough to squeeze between fittings in ships thus preventing leakage. Hyatt, as has already been mentioned, prepared flexible Celluloid by using camphor (a plasticizer) (9). The Second World War stimulated production of *PVC* for war needs and the years following the war saw a great many advancements in the use of plasticizers. Even though a great many studies have since been reported on various aspects of plasticizers, the selection of the proper plasticizer for a particular application is still considered an art.

A recent book by Mellan (29) is devoted exclusively to the behavior of plasticizers and should be consulted for quick references by those in need of authoritative information on the subject. Mellan points out in his text that "the term plasticizer means different things to different users, who apply it to those qualifications which lie within the limits sought by their particular industry." He, however, gives a definition for the term which appears to be adequate for this discussion:

> A plasticizer is generally defined as a substantially nonvolatile, highboiling, nonseparating substance which, when added to another material, changes certain physical and chemical properties of that material. (Ref. 29, p. 4).

One general behavior of the polyvinyl chloride, modified with the addition of the plasticizer, is flexibility and softness. Other physical properties are altered depending upon the particular plasticizer or plasticizers and the concentration present. It is the diversity of physical properties which can result by the proper use of a plasticizer which makes possible the broad range of end uses now seen for *PVC*.

Often two adjectives (internal and external) are used with plasticizers which should now be clarified. The term "internal plasticizer" should be relegated to the copolymers and implies that the ordinary polymer chain has been modified by the inclusion at repeating intervals, in the chain proper, another structural unit which, in

effect, reduces the bond strength between adjacent chains and imparts polymer mobility. "External plasticizers" are those modifying ingredients which are added to the polymer mass but which do not become an integral portion of the chain. These "external" ingredients are meant when one speaks about a plasticizer being added to a plastic material and it will be helpful to all concerned if the term "internal" is not used at all except in the specific instances where copolymers are discussed.

If one were to itemize all of the currently suggested plasticizers, he would assemble an imposing list of ingredients. This large number of ingredients has been extremely difficult to classify and for this reason many different classifications have been suggested. Boyer (30) has given a simple classification by dividing plasticizers into three distinct types: (*a*) the solvent type, (*b*) the nonsolvent type, and (*c*) the polymeric type. Each of these types behaves in a different manner but in effect all reduce the cohesive forces between two or more of the polymer chains thereby imparting chain mobility. Often commercial classifications of plasticizers are more conveniently divided under their chemical types as, for example, glycolates, sebacates, adipates, laurates, stearates, abietates, esters of polyhydric alcohols, etc.

Neither the art nor the growing science in the plasticizer field has produced the one universal plasticizer and it is doubtful if in fact this objective will ever be reached. Undoubtedly newer and better ones will come into being but more for particular applications.

In general, flexible *PVC* items are prepared from a specific formulation which includes a number of ingredients. For example, a common formula for a garden hose may be

Resin—100 parts 95 to 5 vinyl chloride-acetate copolymer
Plasticizer—50 parts dioctyl phthalate
Color—3 parts dye or pigment
Stabilizer—2 parts thermal stabilizer such as dibutyl tin laurate
Lubricant—1 part aluminum stearate
Filler—as required

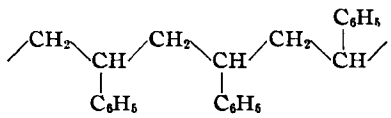
In any formulated plastic, there is always a propensity for one of the ingredients to migrate into the environment which has intimate contact with the material. This can become a serious problem, particularly when the material is used for foods or for drug products. Recent changes in the Food and Drug regulations in regard to foods require that only approved ingredients be included in packaging materials for food. At present there is no such regulation for devices for drug products or in general for plastic items used in medical practice. This point on migration will be discussed again in more detail in a later portion of the paper.

The primary use of the polyvinyl chlorides is in tubings for the administration of blood, drugs, nutritional fluids, and for the collection of blood and other body exudates. Other uses such as flexible containers for blood are in current practice.

Polystyrene (27, 31)

As was mentioned in an earlier portion of this review, polystyrene is one of the oldest plastics known but the real growth is a direct outcome of the Second World War. The sales of styrene-type polymers reached an impressive sales volume of 922 million pounds last year, placing it second to polyethylene in use (25). One of the main reasons for the high volume of use is the low cost per pound as compared to comparable resins to be used for the same application.

Polystyrene is formed from the monomer styrene in the presence of a catalyst and proper conditions to give a long chain configuration which may be represented in its simplest form as



When the term polystyrene is used it indicates that the plastic is composed of the homopolymers of styrene and often this material is referred to as *normal* or *conventional* polystyrene. Other types of polystyrene are also (modified and copolymers) produced, several of which will be alluded to presently.

Polystyrene (conventional) is a hard, amorphous solid, having transparent properties. The material is stable to distortion up to approximately 95° at which point it begins to become pliable, and as the temperature is further increased the material becomes soft and putty-like. Certain modifications in the structure of polystyrene will either increase or decrease the softening temperature. The brittle characteristic of conventional polystyrene may be overcome by combining various concentrations of rubber with the polymer producing the *impact* polystyrenes. Certain desired properties are lessened, however, with these impact plastics, as for example, lack of clarity and decrease of hardness. The shock resistance or toughness of impact polystyrene may be increased by increasing the content of rubber in the material

and often these materials may be further classified as (*a*) intermediate-impact, (*b*) high-impact, and (*c*) super-impact polystyrenes.

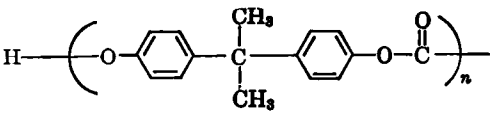
Crystalline polystyrene can be prepared by stereopolymerization following Natta's work and, as with the polypropylenes, isotactic polymers are possible. Commercial use of these stereopolymers is still in its infancy and little practical information is at present available to judge the merits of the material.

Added dimension has been achieved by copolymerization of the styrene monomer with other monomers. In particular, these copolymers are more stable to chemical agents and heat and are finding a long list of special applications. Combination of polystyrenes with fibrous glass makes possible other uses for this material. Finally polystyrene can be made into a foam which can take on any shape desired. The resultant light, porous material has numerous industrial and household applications.

Use in pharmacy and medicine may be listed (in part) as follows: syringes, parts of administration sets, containers, cups, plates, rigid glass-like tubings for various purposes, and other items where rigidity and clarity are desired.

Polycarbonate (32)

Polycarbonates represent a large group of esters of carbonic acid but the most successful material is prepared from the monomer, bisphenol-A [2,2-bis(4-hydroxyphenol)propane]. The structure formula for the bisphenol-A polycarbonate may be depicted as



Commercial production of polycarbonate began in Germany in 1959 and was followed by the United States in 1960. A number of important properties has stimulated a great interest in this material. Commercial polycarbonate has an extremely high molecular weight—weights up to one million have been produced. One of the outstanding characteristics of the polycarbonates is the resistance to physical changes in a broad range of temperatures (−215 to 250–275°). The plastic is known for its dimensional stability, high impact strength, resistance to stain, low water absorption, and transparency. Much of the characteristic properties of the polycarbonates can be directly attributed to the structural configuration of the chains which impart a great deal of rigidity to the molecules. This rigidity is probably due to the rather long monomer units anchored to the pivotal carbons (between two phenyl groups). If the pivotal carbon is replaced with an alkyl group, a definite increase in flexibility will occur. The rigidness of the chains can be further emphasized by comparing the glass transition temperature of this new plastic with several of the other well-known polymeric materials. For example, the glass transition temperature of polycarbonate is 145° to 147° for nylon 66, and −135° for polyethylene. Replacement of the methyl groups on the pivotal carbon with larger radicals increases the rigidity of the chain while substitution with hydrogens increases the flexibility.

Originally it was believed that all of the polycarbonates were completely amorphous but recent studies have indicated that various degrees of crystallinity can be produced. In those samples where crystallinity has been induced the crystallites are usually smaller than the visible wavelength of light and transparency is still maintained even for materials which may be several inches thick.

Polycarbonates are quite stable to degradation by oxygen because of the high degree of aromaticity. Further resistance to oxidation is enhanced due to the absence of secondary or tertiary carbons. The lack of reactive hydrogen atoms in the vicinity of the ester linkages helps stabilize the polymer and prevents degradation or cleavage of the chains. Most solvents do not attack the plastic material even after prolonged periods of time but solvents such as the chlorinated hydrocarbons, cyclic ethers, and tertiary amines will have solubilizing effect.

Polycarbonates have extremely large molecular weights, at times reaching a value of one million. Increase in molecular weight will offer certain advantages for a particular application but a molecular weight range may be reached where further increase in weight will serve no real advantage but, instead, may have a pronounced objectionable effect on several of the properties. As with all plastic substances, molecular weight distribution will influence a number of the properties but not enough experimental data are at hand to delineate the exact influence.

Much use is already being made of this material in both industrial applications and in household applications. The medical and pharmaceutical field is turning its attention to the polycarbonates for such devices as animal cages, parts of surgical equipment which require a window, and replacement of conventional materials where toughness, heat resistance, and transparency are needed.

Nylon (33, 34)

In the late 1920's, W. H. Carothers of Du Pont became interested in synthesizing various types of polyamides. It was, however, in 1938 that Du Pont announced the realization of a new material resembling in many ways wool and silk. This new plastic material was baptized with the generic name nylon and now includes a number of different types of polyamides and copolymers. Nylon has distinctive properties such as high melting point, toughness, resistance to chemical attack, and resistance to abrasion, as well as other qualities which are desired for a number of applications.

In general, polyamides are synthesized by two methods: (*a*) condensation of a diacid with a diamine or (*b*) self-condensation of amino acids. Common to all nylons is the repeating amide linkage running through the chain. At present there are a number of nylon plastics but only four types appear to be enjoying commerical success. These are: (*a*) nylon 6/6, (*b*) nylon 6/10, (*c*) nylon 6, and (*d*) nylon 11. The numbers following nylon indicate that both the acid and amine used for synthesis contain six carbons. Commercial samples of nylon must contain an antioxidant since there will be a tendency for degradation of the polymer in the presence of ultraviolet light and air. Nylons are quite stable to heat but degradation by hydrolysis in an acid medium at high temperature is possible.

The polar sites (referring to the amide groups) are responsible for many of the properties of the nylon. Strong forces of attraction become possible between two parallel chains by hydrogen bonding. In this case, hydrogen atoms from the nitrogens in one chain bond with the oxygen atoms in an adjacent chain. Clearly the more of these bonds per unit of polymer segment the greater will be the force of attraction. Table III lists a number of polyamides with their respective melting points and it can be seen that a great difference exists among the various melting points.

TABLE III.—APPROXIMATE MELTING POINTS (IN AIR) FOR VARIOUS NYLONS[a]

Diamine	Dibasic Acid	M.p. of Nylon, °C.
Ethylene	Sebacic	254
Tetramethylene	Adipic	278
Tetramethylene	Suberic	250
Tetramethylene	Azelaic	223
Tetramethylene	Sebacic	239
Pentamethylene	Glutaric	198
Pentamethylene	Adipic	223
Pentamethylene	Pimelic	183
Pentamethylene	Suberic	202
Pentamethylene	Azelaic	178
Hexamethylene	Sebacic	209
Octamethylene	Adipic	235
Octamethylene	Sebacic	197
Decamethylene	Carbonic	200
Decamethylene	Oxalic	229
Decamethylene	Sebacic	194
Piperazine	Sebacic	153

[a] Floyd, D. E., "Polyamide Resins," Reinhold Publishing Corp., New York, N. Y., 1958, p. 41.

A number of studies have been conducted on the effect that substituent groups have on the property of nylon. It has been shown, for example, that if the hydrogens from the amides are replaced with other groups, *i.e.*, methyl, ethyl, propyl, and amyl, a decrease in molecular weight will occur. These substituted groups not only prevent hydrogen bonding but also by steric effect prevent close approach of parallel chains. This results in nylon having a lower molecular weight, higher solubility, and more flexibility. The same type of alteration in properties may be observed by immersing samples of nylon in phenolic solutions. In this case molecules of the particular phenol will penetrate the plastic and compete for polar sites, thereby breaking existing hydrogen bonds (between two chains). If sufficient concentration of a particular phenol is present, the nylon will dissolve.

The intermolecular forces between the polar sites (of adjacent molecules), as has been stated, holds the polymer chains firmly together but secondary valence forces are also present to stabilize the interaction. It should be obvious that each chain, if highly bonded, will be parallel to its neighbor chain. This gives rise to an orientation of polymer molecules in such a manner that a high state of crystallization becomes possible. Even though other polymers can be drawn with or without addition of heat, the polyamides are a very good example of a plastic material which can be made more crystalline by simply pulling the material. In effect this straightens out the chains and orients the molecules in a parallel direction which permits close spatial contact of the polar sites (through hydrogen bonding) in one chain to polar sites in another. Nylon filament to be used for textile purposes is manufactured as an amorphous polyamide which on cold-drawing up to six or eight times its original length gives a highly crystalline material.

Many industrial uses have been found for nylon such as gears, machined parts which may have to withstand heavy mechanical shock and high temperatures, tires, tubings, instrument casings, and films for packaging oil products and food. In medical and pharmaceutical practice, nylon has been used for syringes, parts of administration kits, containers, and film

packaging of surgical items such as sutures or delicate and expensive surgical items. No doubt the polyamides will find wider use in medical practices as the cost of the material is reduced.

Cellulosics (35, 36)

Under this heading belong a number of thermoplastic products having considerable commercial value as packaging material, especially of the film type. For example, the following would fall under the so-called cellulosic-type plastics: cellulose acetate, cellulose acetate butyrate, cellulose propionate, cellulose nitrate, and cellophane. Each material may find advantages over the others for a specific application. These groups of materials have apparently reached a plateau in yearly consumption which should remain about constant for the next several years. Approximately 141 million pounds of cellulosics were consumed in 1962 (25).[2]

Cellulose nitrate, as has already been mentioned, is the oldest plastic material which has found successful commercial application. In an attempt to find a less flammable material than celluloid, cellulose acetate was developed in the early part of this century and as early as 1912 cellulose acetate was used in photographic film. True commercial success of this material, however, did not take place until around 1927. Since then, the other cellulose esters have been developed.

The various cellulosics are prepared from purified cotton linters or wood cellulose which contain high content of alpha cellulose. Each cellulose molecule has approximately 3000 glucosidic units, each unit having three available hydroxyl groups. It is the esterification of these hydroxyl groups with a particular agent which gives rise to a specified plastic.

Cellulose Acetate.—This cellulosic-type plastic has numerous uses today from such items as ladies' shoe heels to transparent windows in envelopes and cartons. The material is tough and has high impact strength. A clear transparent material can be prepared and a variety of colored items are possible with the use of cellulose acetate. The material has advantages over some of the other thermoplastics because of its dimensional stability. The flexible plastic must contain various proportions of plasticizers. In pharmacy and medicine the most use is being made of this material for one form or another of film packaging.

Cellulose Acetate Butyrate.—This material has many of the properties of cellulose acetate but appears to have improved dimensional stability. It tends to absorb less water than its close cousin and, because higher boiling plasticizers can be used, less plasticizer is needed in a cellulose acetate butyrate formula than for cellulose acetate. These two factors aid in producing a more stable material. It is imperative that cellulose acetate butyrate not be contaminated with other plastic materials since this may cause a diminution of the quality of the item being produced. A variety of uses have been found for this particular material, especially for items which will be exposed to various weather conditions. In the pharmaceutical field it is used as a packaging medium, particularly for blister and skin packaging.

Cellulose Propionate.—This is an extremely tough plastic and requires approximately half the quantity of plasticizer which must be added to cellulose acetate for a particular application. The use of this material for parts of equipment and instruments is due to its toughness and durability to shock.

Cellulose Nitrate.—This plastic is considered the toughest of the thermoplastic materials. A number of disadvantages, however, have decreased the use of this material with the advent of the other cellulosics and newer thermoplastics. The chief disadvantages are its flammability and tendency to become brittle and to discolor in the presence of light.

Regenerated Cellulose or Cellophane.—This substance is not considered as a plastic material even though, often for convenience, it is included as such. We will also include it here with the cellulosics since it has a very close relation to the other materials, being a product of cotton or wood. The use of cellophane as a film packaging material is well known and probably this material has had more applications for a variety of items than any other packaging material. In the late 1920's, a process was patented by Du Pont which made possible moisture-proof cellophane. It was this event which ushered in the transparent flexible packaging industry.

Silicones (37)

The silicone products came into commercial use during the Second World War even though they were known as laboratory curiosities in the last century. Properties such as stability to high and low temperatures, resistance to oxidation, water repellency, and unusual inertness have aided the growth of these materials for a number of special applications where use of other plastic materials is not always possible.

The silicones are a group of materials which

[2] Note that this does not include cellophane.

are composed of a molecular backbone of alternating atoms of silicon and oxygen. Organic groups are attached to this backbone in repeated intervals. The silicones can be produced to yield physical properties from various viscosities as a liquid to a solid material. They can be used as coating material for glass and other materials and as encapsulating agents. Other uses, too numerous to mention in this short résumé, have also been found which give great versatility to the silicones. The advent of silicone rubber has made it possible to expose tubing to very extreme temperatures without altering the physical or mechanical properties of the material. Many uses have been found for the silicones in pharmacy and medicine. The coating of glassware for water repellency is now widely used in most laboratories and by the pharmaceutical industry for bottles and ampuls. Surgeons are finding a great deal of success with the silicone rubbers as prosthesis for various segments of the body. No doubt more and varied uses will be found for this group of plastics in the near future.

Others[3]

Some consideration has been given to a number of plastics but it should be kept in mind that there are other important plastic materials which may have individual advantages for specific applications in both pharmacy and medicine. One would not be doing justice to plastics in general if mention were not made of the *acrylics* which have been employed for a great period of time in the field of dentistry. The *acetals* are of more recent vintage and should find favorable use as parts of equipment which previously were made from metals. A number of *fluorocarbons* have been introduced since 1943 when the first successful material was manufactured. These materials have good thermal and chemical resistance and are finding uses in medical practice as tubings, synthetic prosthetic devices, and replacement for rubber and metals where good wear is desired. The *isocyanates* or, perhaps more correctly, the *polyurethanes* are an interesting group of materials. Various shapes and sizes of flexible foams can be prepared from polyurethane which take on the characteristics of rubber or rigid foams can be produced for special applications. These materials are finding use in surgery as prosthesis and as packing material for certain pharmaceuticals or agents which need extreme care in shipment. *Polyvinyl alcohol* behaves quite differently from most of the other plastic materials. This particular material will dissolve when placed into hot water. Use is now being made of this unique property to design packets which will contain a product to be eventually dissolved in water. All that needs to be done is to place the packet into warm water and, in a brief interval of time, the container will dissolve, releasing its contents. Measured amounts of ingredients can thus be assured without the need of the user to do the measuring or even to come in direct contact with the product. A group of polymeric materials which range from brittle solids to low viscosity liquids are the *epoxies*. These compounds are used as coating materials and in cementing various types of materials with such adhesion that often the components will break before separation at the cement point.

[3] See "Modern Plastics Encyclopedia" (1962 issue) for further information on plastics.

DRUG-PLASTIC CONSIDERATIONS

Introduction

In the previous section devoted to general information, the reader had an opportunity to explore, in a mild manner, a number of insoluble polymeric materials collectively called plastics. Even though the discussion on the selected plastics was brief, it should be evident that a large number of materials exist which have properties quite different from each other. Furthermore, it has been pointed out that even a plastic material having the same generic name can be dramatically different from the same named material from another source. Added to this already complex picture are the numerous modifications which are possible in formulating a final plastic material. From this maze of materials, often not clearly defined as to specifications, the pharmaceutical scientist must select one or more materials for a specific application. In the same manner, the surgical house must, with the utmost caution, find the proper plastic material for the numerous devices presently entering the hospital field in part or whole of a polymeric material.

The task of selecting the "right" material for a specific application was at one time given little attention in the pharmacy and medical fields. A prior assumption was made that plastics were quite inert and no serious problems could develop. It soon became evident that this was not the case and interest and energies were directed to evaluate materials as to their pharmaceutical and medical acceptability. Information and guidance were solicited from the various segments of the plastic industry to help formulate materials and devices which would prove

of equal quality to those standard materials which have been used for countless decades. Even this approach, it was soon found, was not enough since applications to pharmacy and medicine required special studies which had not been conducted by the plastic industry and which, it appeared, would have to fall on the shoulders of both the pharmaceutical industry and the surgical houses manufacturing and distributing devices for medical practice. A number of firms, representing both groups, are now pursuing an active and conscientious research program to eliminate possible problems in the use of plastics or to at least circumvent these problems to the advantage of all.

The point has been reached to become more specific in this review and discuss those problems which have or may become of consequence to both the pharmaceutical worker and to those other individuals and groups who will, in one manner or another, be involved in the chain of events leading to the ultimate use of the plastic item. In particular, the attention of the reader will be directed toward drug-plastic problems. Since plastics are relatively new to the practice of pharmacy and medicine, it will be convenient to classify these considerations in a rather arbitrary manner to facilitate the presentation. For this reason drug-plastic consideration is subdivided into five parts: permeation, leaching, sorption (including adsorption and absorption), chemical reactivity, and alteration in the physical properties of the plastic. As will be noted in due time, certain aspects of the various problems will be common to more than one class or section.

Permeation

One of the chief advantages of glass containers, for pharmaceutical solutions, is the lack of penetration of molecules from the solution in and through the glass walls or, conversely, the entrance of gas molecules through the glass wall into the solution. With plastic materials, one is immediately confronted with the problem of permeation in two directions: (*a*) from solution through the plastic into the ambient environment or (*b*) from the ambient environment through the plastic into the solution. It should be obvious at this point that the permeation rate will depend primarily upon the particular plastic material used (see Table IV).

Theory.—When a gas or a vapor is placed on one side of a plastic film, molecules of the gas will tend to dissolve at the surface of the film and will diffuse under a concentration gradient through the film reaching the other side (or the low pressure side) of the film. After a short period of time a steady state will be reached whereby the gas will diffuse through the film at a constant rate, providing that a constant pressure difference is maintained across the film.

TABLE IV.—TYPICAL GAS TRANSMISSION RATES OF PLASTIC FILMS AT 23° C.[a]

Plastic Film	Gas Transmission Rates, ml./m²—24 hr.—1 atm.) O_2	N_2	CO_2
Cellulose acetate	350	1500	7,800
Methylcellulose	1300	450	6,800
Polyethylene			
0.917	2700		
0.950	1700		
0.960	1600	440	...
Polyethylene terephthalate	50	8.4	240
Polystyrene	4500	640	11,000
Polyvinyl chloride, plasticized	190–3100	58–810	430–19,000
Polyvinyl chloride, rigid	120	20	320
Polyvinyltoluene	5700	1200	17,000
Rubber HCl	390	62	1,100
Saran	16	2.5	50
Styrene-acrylonitrile (copolymer)	900	120	2,800

[a] Brown, W. E., and Sauber, W. J., *Mod. Plastics*, 36, 107 (Aug. 1959).

In order to develop the usual equations for diffusion, consider a film having a thickness of l cm. and of unit area (38). One side of the film will be a pressure P_1 (high pressure side) while on the other, a pressure of P_2 (low side) will exist. A schematic representation of this situation is shown in Fig. 3.

For simplicity at layer A of the film, a concentration of gas equal to c_1 will be present while in the last layer (at B) the concentration will be c_2. The letter x will designate a distance between

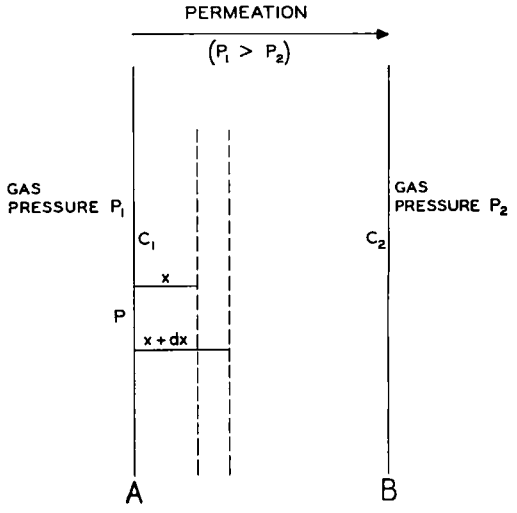


Fig. 3.—Representation of a gas entering and passing through a film from surface A to B.

two planes in the film, and $x + dx$ another distance between two planes. Designating the rate of gas permeation at x as q ml. per second and permeation at $x + dx$ as $q + (dq/dx)dx$, the amount retained per unit volume will equal $-(dq/dx)$ which can be equated to the rate of change of concentration c with time or

$$-\frac{dq}{dx} = \frac{dc}{dt} \qquad \text{(Eq. 3)}$$

When a steady-state condition is reached, $dc/dt = 0$ and q will become a constant.

No permeation will occur when the concentration gradient is zero and extremely high rates of permeation when the gradient is very large. The mathematical relationship between permeation and the gradient of concentration can be expressed by Fick's first law or

$$q = -D\frac{dc}{dx} \qquad \text{(Eq. 4)}$$

where D is defined as the diffusion coefficient or constant. Fick's second law as an equation may be developed by combining Eq. 3 with Eq. 4 to get

$$\frac{dc}{dt} = \frac{d}{dx}\left(D\frac{dc}{dx}\right) \qquad \text{(Eq. 5)}$$

If the diffusion constant, D, is independent of concentration, Eq. 5 reduces to

$$\frac{dc}{dt} = \frac{Dd^2c}{dx^2} \qquad \text{(Eq. 6)}$$

At a steady state of diffusion the rate of permeation, of q, will be constant and by integrating Eq. 4 between the two concentrations, c_1 and c_2, Eq. 7 will result

$$q\int_{x=0}^{x=l} dx = -D\int_{c_1}^{c_2} dc$$

or

$$ql = -D(c_2 - c_1) = D(c_1 - c_2)$$

$$q = \frac{D(c_1 - c_2)}{l} \qquad \text{(Eq. 7)}$$

Henry's law relates the concentration of gas at each surface in the film to the partial pressure of the gas or

$$c = S \cdot p \qquad \text{(Eq. 8)}$$

where S is the solubility coefficient of the gas in the film at equilibrium and p the partial pressure. Substituting $S \cdot p$ for the concentration terms in Eq. 7 will give Eq. 9

$$q = \frac{DS(p_1 - p_2)}{l} \qquad \text{(Eq. 9)}$$

The permeability constant, P, can now be defined as $P = D \cdot S$ or

$$P = \frac{q \cdot l}{(p_1 - p_2)} \qquad \text{(Eq. 10)}$$

If a plot is made of q *versus* time for a particular material, as appears in Fig. 4, it will be noted that a short time period must pass before a linear relation is reached. This portion of the curve indicates that a steady state of diffusion has been reached. The initial time period (non-linear portion of curve) is considered the non-steady state and Fick's second law applies (Eq. 5 or 6). No true solution for this equation for a finite solid has been developed, but for those cases where the diffusion coefficient is independent of concentrations and boundary requirements are stated, a number of mathematical approaches have been postulated. Of these, the works of Daynes (39) and Barrer (40) are perhaps the most widely accepted.

The equation which has found considerable use in the evaluation of D, P, and S is Barrer's equation (40), often referred to as the "time-lag" equation or method, which may be stated as

$$D = \frac{l^2}{6\tau} \qquad \text{(Eq. 11)}$$

where l is the thickness of the film and τ is referred to as the "time lag" which may be found by extrapolating the linear portion of the curve (see Fig. 4) to the time axis. This value, τ, may then be used to calculate D in the above equation. The permeability constant, P, can be evaluated from the slope of the linear portion of the line. Since D and P are known it is a simple matter to find S.[4]

As may be expected, temperature will have a direct effect on D, S, and P. Experimental data at various temperatures will follow the Arrhenius relationship and thus

$$\begin{aligned} D &= D_0 \exp.(-\Delta E_D/RT) \\ S &= S_0 \exp.(-\Delta H/RT) \\ P &= P_0 \exp.(-\Delta E_p/RT) \end{aligned} \qquad \text{(Eq. 12)}$$

where D_0, S_0, and P_0 are pre-exponential factors, ΔH is the heat of solution, ΔE_D the activation energy for the diffusion, and ΔE_p activation energy for permeation.

Studies of transmission of gases through permeable materials are not new. Graham, in the 1860's, had investigated the penetration of gases through rubber. Greater emphasis on these types of studies increased with the development of the synthetic materials for both theoretical and practical reasons.

[4] D = cm.²/sec.; P = ml.(S.T.P.)mm./cm.²/sec./cm. Hg; S = Gm./Gm./cm. Hg. It should be noted that other units have and may be used for these three terms.

Measurement of Permeation.—A variety of methods for studying gas permeability through plastics have appeared in the past decade (41–44). It is apparent that measurements of this type are not always quite as simple as may be thought. Major and Kammermeyer (45), in a recent publication, cite that they have finally reached the conclusion, after thousands of permeation measurements, "that they are anything but simple and often can lead to frustrating experiences." This perhaps is one reason why permeation data often do not agree when different laboratories have conducted studies on permeation on the same type of material. Keeping in mind that problems may arise in permeation studies, it is still possible to list a number of general methods which have been used. For this listing it is convenient to refer to the work of Brown and Sauber (46) who mention five methods of measuring permeation: thermal conductivity, refractive index, mass spectrometry, gas analysis by chemical means, and pressure-volume-temperature (*PVT*) methods. Of these, the last method appears to be the most practical and, in fact, is used as an *ASTM* test for gas transmission rate of plastic sheets (47).

In general, the *PVT* method employs a cell which contains two compartments separated by the particular sample of film to be studied. Gas is introduced to one side of the film and the pressure change on the other side recorded over a period of time. From these data the volume of gas transferring from the high pressure side to the low pressure side may be calculated, which in turn can be used to calculate the permeation rate or constant. Brown and Sauber (46) developed the type of instrument which is now included in the *ASTM* test, referred to above. This instrument lacks high accuracy if very exacting permeation data are needed, but serves to give acceptable data when rapid measurements are needed as might be the case for control work or for other industrial applications. The already mentioned authors, Major and Kammermeyer (45), suggest a new instrument which operates basically in the same manner as the *ASTM* instrument, but which seems to give greater accuracy without the loss of speed.

For more precise and accurate permeation data using a *PVT* method, it will be necessary to set up a more elaborate instrument such as used by Stannett and co-workers in their numerous permeation studies (48–51). Certain advantages, however, can be gained by the use of this latter instrument since the accumulated data can then be calculated to give both the solubility and the diffusion constants (see Fig. 4). The chief disadvantage of Stannett's instrument is the painstaking techniques which are needed for proper operation.

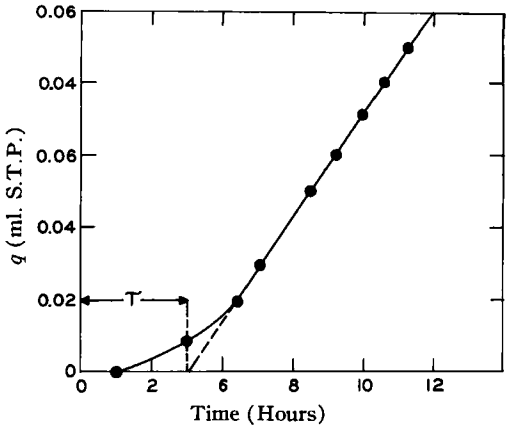


Fig. 4.—Plot of gas transmission *vs.* time through a plastic film.

Penetrant Molecules.—*Permanent Gases.*—The particular plastic material will influence the permeation rate of a gas as may be noted by referring to Table IV. Even for the same generically named plastic, the rate may differ due to such factors as molecular weight, molecular weight distribution, branching, degree of crystallinity, and the presence of other ingredients. Some of these factors have been investigated in some detail, but a great deal still has to be done to have a true indication of the influence a combination of these factors has on the penetration of gas molecules. One must be content to examine several of the factors individually, keeping in mind that other forces are also exerting an influence even though these may not be so apparent.

From simple theoretical considerations gas molecules must find "holes" in the plastic material to travel through the material. Any hindrance to this passage will of necessity tend to decrease the permeation rate. Polymeric materials, which have a great deal of crosslinking, will retard the movement of the gas molecules. Crystallite formation and the degree of crystallinity in the plastic material will have a very appreciable effect on permeation and it is now generally accepted that gas molecules probably travel through the amorphous zone. High crystalline materials would thus have low rates of permeation.

Considerable work has been done on the influence of crosslinking in rubber on permeation of various gases (52, 53). Less information is available on the crosslinking of plastics, but it

appears that permeation follows in somewhat the same general order as seen for rubber—the more crosslinking, the less permeation (54).

Table V is taken from the work of Rogers, Meyer, Stannett, and Szwarc, and indicates the influence of crosslinking on the permeability of unirradiated and irradiated polyethylene to three gases. The higher dosed plastic showed a reduction in the permeation rate revealing an appreciable increase in crosslinking, whereas the lower dosed material apparently had little change in the crosslinking, as may be surmised from the lack of a significant change in the permeation rate.

Many of the currently used plastic materials for packaging purposes have various degrees of crystallinity. As has been indicated, the greater the degree of crystallinity the less will be the permeation rate. This is an oversimplification of a rather complex phenomenon, but a number of authors have attempted to describe the influence of crystallinity on the permeation rate (55, 56). An example of the effect of crystallinity on permeation may be noted by observing Table VI. With both polyethylene and polychlorotrifluoroethylene with the three gases, a decrease in permeability takes place as the approximate crystallinity increases.

It has been theorized that a decrease in permeability of permanent gases in materials of high crystallinity is probably due to the decrease in the diffusion constant, since little change occurs in the solubility constant of the gas in the material. At one time, it was believed that the diffusion constant was related by simple proportion to the amorphous content of the plastic (57). This has since been proved as false (56). Other reasons influence the diffusion constant of the gas. Crystallite zones may have an effect on the geometric structure of the amorphous zones, giving rise to difference in diffusion and, of course, on permeability. Michaels and Parker clearly state that diffusion of a gas in polyethylene is not solely a function of the degrees of polymer crystallinity, even though it is highly dependent upon it (58). They point out that the crystallite shape has an influence in altering the diffusion which, in turn, alters the permeability.

Several investigators have indicated that polyethylene permeability can be related in a linear fashion to the density of the material (59, 60). This prediction seems not to have been verified by Brandt (61). Lasoski and Cobbs (62) postulate that permeation will be more apt to be related to the square of the density of the material. Alter (63) in a critical investigation of permeation and using the previous concepts of Myers, *et al.* (64), Klute (59), and Lasoski and Cobbs (62) developed the simple expression $P = K(1 - d)^n$, where K is a constant characteristic of each gas, n is an exponential value without further definition, and d is the density, for relating permeation to

TABLE V.—PERMEABILITY CONSTANTS FOR POLYETHYLENE (INFLUENCE OF CROSSLINKING)[a]

Gas	Temp., ° C.	Unirradiated	Irradiated	
			10^7/rad	10^8/rad
Nitrogen	0	2.59×10^{-10}	2.67×10^{-10}	1.46×10^{-10}
	15	7.84	7.72	4.36
	30	21.5	20.1	11.0
	45	54.6	50.6	27.4
Oxygen	0	11.0	...	5.91
	15	27.5	...	15.3
	30	69.4	...	34.8
	45	143	...	73.7
Carbon dioxide	0	54.7	54.6	29.7
	15	130	129	72.7
	30	280	277	152
	45	540	542	287

[a] In part: Rogers, C. E., Meyer, J. A., Stannett, V., and Szwarc, M., "Permeability of Plastic Films and Coated Papers to Gases and Vapors," Tappi Monograph Series No. 23, New York, 1962, p. 20.

TABLE VI.—EFFECT OF CRYSTALLINITY ON GAS PERMEABILITY[a]

Polymer	Approximate Crystallinity, %	P, at 30° C.		
		N_2	O_2	CO_2
Polyethylene	60	1.9×10^{-9}	5.5×10^{-9}	25.2×10^{-9}
	69	0.66	2.1	7.4
	81	0.27	1.1	4.3
Polychlorotrifluorethylene	30	0.008	0.05	0.11
	80	0.004	0.013	0.03

[a] Myers, A. W., Rogers, C. E., Stannett, V., and Szwarc, M., "Permeability of Plastic Films and Coated Papers to Gases and Vapors," Tappi Monograph Series No. 23, New York, 1962, p. 53.

density. Experimental data for nitrogen, oxygen, and carbon dioxide in various film samples of polyethylene having different densities followed in general the mathematical expression shown above. Alter (63) believes that there is more justification in relating permeability to density of the material than to the usual degree of crystallinity or to the volume fraction of the amorphous zone.

Organic Vapors.—The solubility coefficient and the diffusion coefficient are constants at any given temperature for penetrant gas molecules. Since the permeation coefficient is a product of the solubility and diffusion, P will also be a constant. Large permeation constants for a specific gas are primarily dependent upon an increase in the diffusion constant while, in most instances, for organic vapors, an increase in the permeation rate is much more dependent upon an increase in the solubility of the vapor molecule. The ability of a polymer to attract and sorb (increase in solubility) penetrant vapor molecules will obviously be related to the physical and chemical properties of both the penetrant and the polymer. As a first approximation then, it can be assumed that as the chemical structure of the penetrant becomes more similar to the polymer, greater solubility will result and as a consequence greater permeability. This has in fact been found to be generally true. For example, Bent and Pinsky (65) found that permeation through polyethylene increased in the following order: alcohols, acids, nitroderivatives, aldehydes, ketones, esters, and hydrocarbons.

Since very little interaction occurs between the permanent gases and a particular polymer, the permeability rate is not concentration dependent. Quite different, however, is the case for most of the vapors since many of these will interact (sorb) with the polymer. Increases in concentration of the penetrant in the plastic will usually have a material effect upon the diffusion. Part of this effect may be due to the ability of the penetrant when sorbed to swell the plastic or to act as a plasticizing agent, thereby permitting greater degree of traversity or diffusion in the material. Often unusual or anomalous results are seen which become difficult to resolve on theoretical grounds.

There has been little success in relating, quantitatively, organic vapor permeation with crystallinity, amorphous content, or plastic density, but by indirect means, data have been accumulated at least to support the contention that certain vapors will follow a direct relationship with the amorphous content of the plastics. Perhaps the best example to illustrate this point is the correlation of the data on methyl bromide sorption (consider solubility and sorption as being nearly identical) with the amorphous content in several polyethylenes (51). The top portion of Fig. 5 represents the sorption isotherm in three polyethylenes, each having a different density. As may be noted from this figure, the sorption decreases as the density of the material increases. The single curve at the bottom of the same figure has been recalculated on the basis of the amorphous content of the samples and it will be noted that all the points now fall on the one line. Other molecules, especially those molecules which are considered as good solvents for the plastic, will not show the single curve after recalculation on the basis of the amorphous content (51).

Crosslinking in a plastic material usually will decrease the permeation rate, more probably due to hindering the travel of the penetrant (diffusion) than to decreasing solubility. For those penetrants which are better solvents for the plastic, both the solubility and diffusion will increase slightly at low temperatures, but at higher temperatures the crosslinks prevent the usual swelling and decrease the mobility of the chains and, in consequence, both solubility and diffusion decrease.

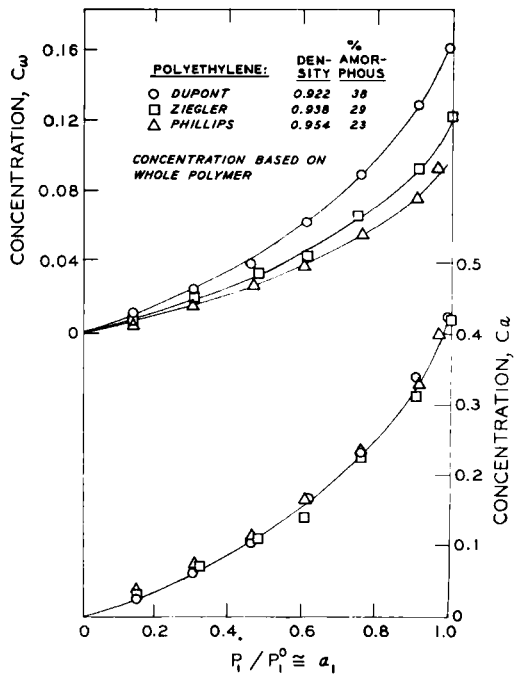


Fig. 5.—Sorption isotherm for polyethylenes and methyl bromide to 0°C. [From Rogers, C. E., Stannett, V., and Szwarc, M., *Tappi*, **44**, 715 (1961).]

As has already been noted on organic vapors, solubility plays an extremely important role in the permeation phenomenon. Reference has also been made that the chemical structure of the penetrant will either increase or decrease the solubility in a particular plastic. One other factor must now be brought to light in permeation of organic vapors: the geometry of the particular organic molecule. It has been found, for example, that as the size and the shape of the penetrant molecule increases, a similar increase in solubility will occur. Figure 6 illustrates a linear relationship between solubility and the molar volume of various penetrants. Solubility (at zero concentration of penetrant) appears also to increase as the cross-sectional area of the penetrant increases. The overall effect upon permeation is not as great as might be expected, since diffusion decreases as the volume and shape of the penetrant increases. Other factors, of course, are involved and for this reason it becomes quite difficult to predict the behavior of certain organic vapors in a particular plastic material.

In general, permeation will usually increase with an increase in temperatures. For the permanent gases, the permeability-temperature relationship follows the accepted Arrhenius relationship. With organic vapors, however, this may or may not be the case, depending upon the vapor pressure. For example, it has been found that certain vapors (at a definite vapor pressure) will show a gradual decrease in permeation as the temperature is lowered until a critical temperature is reached, at which point further decrease in temperature will reveal

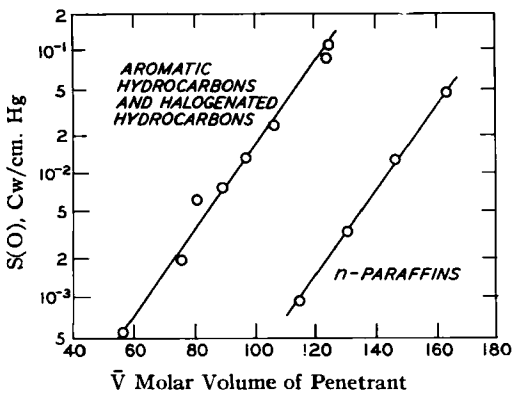


Fig. 6.—Effect of molar volume of penetrant on S(O); polyethylene density 0.922 Gm./ml. at 25°C. NOTE: S(O) is the intrinsic solubility coefficient for a penetrant of zero molar volume. [From Rogers, C. E., Stannett, V., and Szwarc, M., *Tappi*, **44**, 715(1961).]

an increase in permeation (51). From what has been discussed on vapor pressure permeability, it may be seen that the phenomenon is not a simple mechanism and will be altered by many factors, each having some part in the overall mechanism.

Water Vapor.—Water vapor transmission through plastic materials, like a number of the organic vapors, is quite complex and often the results are difficult to interpret. Earlier measurements on water vapor permeability have been found to be in error because of the presence of dissolved air in the vapor (66). The method of evaluating the diffusion constant by the time-lag method will also lead to serious errors (67). Myers, *et al.* (67), found it necessary to calculate the diffusion constant from sorption and permeation data. The rate of permeation is, as might be expected, dependent upon the physical and chemical properties of the particular plastic film. In general, it is possible to describe two general types of permeation: (*a*) those that are dependent on pressure and (*b*) those that are independent of pressure. Past experience has demonstrated that the pressure-dependent permeation follows for most of those polymeric materials which are hydrophilic in nature, such as cellophane, nylon, and polyvinyl alcohol. while the pressure-independent permeation is seen for the hydrophobic polymers, such as the polyethylenes (see Fig. 7).

Pressure dependent permeation (*i.e.*, with nylon) will show large rates of permeation as the vapor pressure increases. This result is due to the ability of the nylon to attract water vapor molecules which are then sorbed to the various polar sites. As the concentration of water in the nylon increases, a swelling and plasticizing effect takes place, which in turn aids the diffusion process. Since both the solubility and the diffusion constants are increasing, the final permeation constant will also be increased. An unusual effect may also be noted with nylon, which in a manner has already been mentioned with certain of the organic vapors (67). For example, an increase in temperature after a certain point will show a decrease in permeation (see Fig. 8). This may be explained in the following way. As the temperature is decreased at a fixed vapor pressure, the relative humidity increases. This increase in humidity is reflected by an increase in solubility in the nylon which accelerates the diffusion process to a much greater extent than the decrease in diffusion normally attributed to a reduction in temperature.

Permeation through the hydrophobic plastics (*i.e.*, polyethylenes) for the most part are

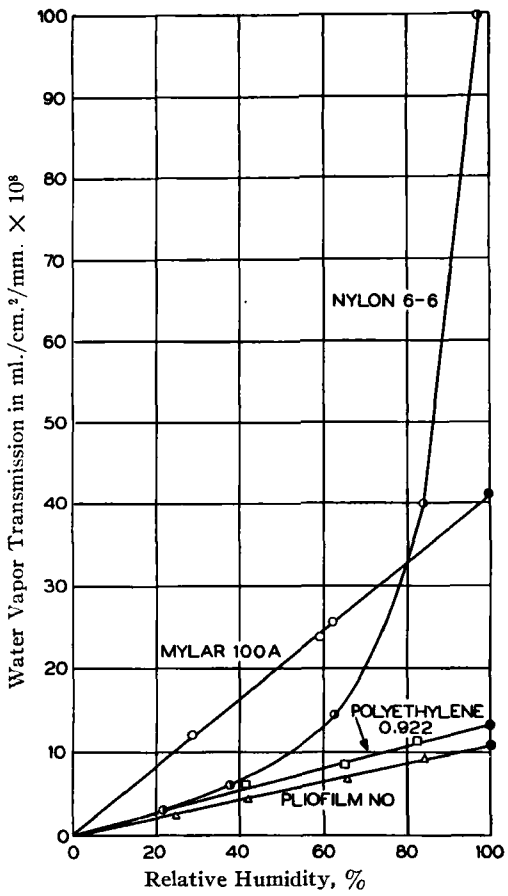


Fig. 7.—Water vapor transmission as a function of humidity for various plastic films. [From Myers, A. W., Meyer, J. A., Rogers, C. E., Stannett, V., and Szwarc, M., *Permeability of Plastic Films and Coated Paper to Gases and Vapors*, Tappi Monograph No. 23, 1962, p. 62.]

independent of the relative humidity. The mechanism for this type of permeation, as pointed out by Myers, *et al.* (67), is not quite as simple as it would at first appear. Strong experimental evidence is at hand to reveal that solubility increases with an increase in pressure, but that the diffusion decreases with an increase in concentration. One apparently compensates for the other, leading to a pressure-independent permeability constant.

Permeation Through Containers.—*Permanent Gases.*—In the discussion which has so far been presented on permeation of permanent gases, organic vapors, and water vapor, the plastic material has been in the form of a film which was then placed in an appropriate apparatus for the study of the penetration and transmission of the gaseous molecules. The parameters of the experiments were kept as simple and as constant as the experimental procedure warranted. Even in these instances, however, as has been demonstrated, a number of anomalous results occurred. For the most part, these data with proper assessment have been utilized by various investigators to give theoretical treatments for penetrant-polymer interactions. Even though this information can serve as a useful foundation for those interested in permeation, certain problems of a more practical nature must still be handled by more empirical means, since the science still has not caught up with the art of manufacture of certain types of plastic devices. This appears to be true for the present plastic containers used in pharmaceutical practice.

Studies of permeation in the intact container for various gases appear not to have been considered in too serious a vein by those presently manufacturing plastic containers. Often the complete formulation of the plastic material, the actual handling of the material, and the exact history of the manufacture of the containers are not really known by the one utilizing the containers. It is possible that any change in a procedure from the acquisition of the raw materials to the final ejection of a completed container will have an influence on the permeation rate of gases. More time and space

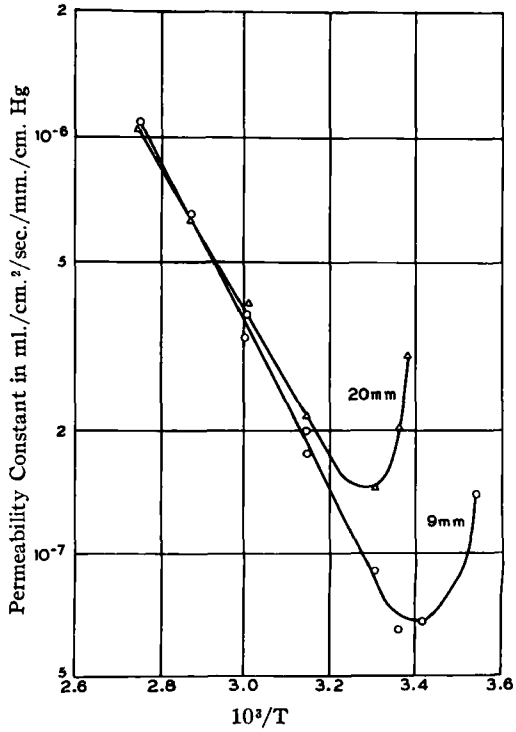


Fig. 8.—Temperature dependence of the permeability constant for water in nylon 66 at vapor pressures of 9 and 20 mm. Hg. [From Myers, A. W., Meyer, J. A., Stannett, V., and Szwarc, M., *Tappi*, **44**, 58(1961).]

than presently available would be needed to explore the various factors which might alter the container to permeation.

Let us for a moment consider some consequences of gas transmission through the walls of a plastic container into a particular drug system. For those agents which are prone to oxidation, a rapid degradation may take place before the useful shelf-life of the product has been reached. Various product development laboratories have noted this effect with one or more of their drug products. Often, even the product development groups have not properly assessed their product in a plastic container to the embarrassment of the company, since reports start arriving indicating that a change in color or a precipitate has formed when the pharmacist, medical practitioner, or patient is about to utilize the medication. Oxygen or air perhaps is the most serious offender in destroying the product, but at times the transmission of carbon dioxide alters the pH of the preparation enough to catalyze degradation by either an oxidation or hydrolysis mechanism. Other problems may be seen due to the transmission of ambient gases, either inside the container or outside. For example, distortion of the container, either collapsing or bulging of the bottle, may be attributed to the transmission of air either out of the container or into the container. Even though there may not be any adverse effect upon the therapeutic efficacy of the medication, the distorted appearance of the container will certainly not add prestige to the company's name.

Vapors and Liquids.—Since 1950 a number of investigators have directed their attention to studying advantages and disadvantages of plastic containers (68–75). Perhaps the most work has been done on regulation-size bottles composed of one of the polyethylenes. Invariably these studies include filling a bottle with a specific ingredient (liquid), stoppering, and exposing it to various temperatures for short and long periods of time, while noting the loss or gain in weight. Pinsky, Nielsen, and Parliman (68) have reported in one study the results of 67 typical chemicals which can be represented by one of the following groups: (*a*) inorganic acids, (*b*) alkalies, (*c*) organic acids, (*d*) alcohols, (*e*) polyhydric alcohols, (*f*) esters, (*g*) ethers, (*h*) aldehydes, (*i*) ketones, (*j*) anhydrides, (*k*) terpenes, (*l*) nitrogen-containing compounds, (*m*) aliphatic hydrocarbons, (*n*) aromatic hydrocarbons, (*o*) chlorinated hydrocarbons, and (*p*) miscellaneous. In studies of this type, it is usually found that for the same material, liquid permeability will invariably be higher than vapor permeability. Table VII will show this trend, taken from the work of Martinovich and Boeke (76). Even though theoretically it is assumed that permeability should vary inversely with the thickness of the plastic, in actual practice, using bottles, this may not be the case.

TABLE VII.—VAPOR AND LIQUID PERMEABILITY OF MARLEX 50[a] AT 80° F.[b]

Chemicals Used	Vapor Permeability[c]	Liquid Permeability[c]
Amyl alcohol	0.353	0.268
Ethylene glycol	0.669	0.873
Acetic acid	0.940	0.993
Aniline	1.09	1.27
Ethyl alcohol	1.26	1.77
Oil of peppermint	1.36	1.75
Butyric acid	1.54	3.61
Benzaldehyde	2.45	3.92
Methyl salicylate	3.39	3.92
Methyl ethyl ketone	4.12	8.05
Amyl acetate	5.54	6.75
Diethyl ketone	7.67	7.67
Ethyl valerate	8.11	9.69
Butyraldehyde	9.86	12.58
Ethyl acetate	13.7	13.7
n-Butyl ether	19.3	34.3
Butyl iodide	52.04	112.72
Heptane	54.0	69.7
Butyl chloride	69.63	86.05
Butyl bromide	76.15	113.19

[a] Trademarked name for Phillips' high density polyethylene. [b] Martinovich, R. J., and Boeke, P. J., "Technical Information on Marlex," Sales Service Lab., Phillips Petroleum Co., Bartlesville, Okla., Oct. 1957, p. 2. [c] Expressed in Gm., 24 hr., 100 in.^{2}, mil thickness.

Results of studies have also revealed that formulations containing volatile ingredients might change, when stored in plastic bottles, because one or more of the ingredients are passing through the walls of the container (77). Often the aroma of cosmetic products becomes objectionable due to preferential transmission of one of the ingredients, or the taste of medicinal products changes for the same reason (78). The plastic container may also have an influence upon the physical system making up the product. For example, certain water-in-oil emulsions cannot be stored in the hydrophobic plastic bottles, since there is a tendency for the oil phase to migrate and diffuse into the plastic.

Heise, Parliman, and Pinsky (73, 74) have done considerable noteworthy work on plastic containers and have made a number of suggestions on how to select a particular container for a general type of product. Even more noteworthy, however, is the series of tests their group has developed to help interested parties evaluate the merits of a container. They include the following tests (74): environmental stress-crack test, using Igepal CO-630 water solution,

impact test, leakage test, side-wall distortion test, and side-wall rigidity tests. Unfortunately, as stated earlier, there is little published information on drug products in plastic containers and it appears that one must study the specific product in each container before final evaluation of the container is possible.

One must not deal lightly with permeation in plastic containers. Some plastic materials will, of course, act as stronger barriers to permeation than others. It is now well known that the high density polyethylenes will give greater protection than the low density types. It has also been found that special types of coating can further prevent permeation. From all past and present indications, it appears that plastic containers for the pharmaceutical industry will have to be manufactured under very special and rigid conditions, a situation which at present, at least for the most part, is not true.

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X-ray and Crystallographic Applications in Pharmaceutical Research II

Quantitative X-ray Diffraction

By JOHN W. SHELL†

In a comparison of quantitative methods, bioassays have an advantage of being specific for an intact molecule. Such assay methods, however, often lack a high degree of precision. Chemical methods usually offer high precision but are notoriously nonspecific. A general quantitative method which offers a combination of specificity and precision is highly desirable, particularly for use in describing pharmaceutical systems. This unique combination is found by the adaptation of quantitative X-ray diffraction to systems containing crystalline ingredients. This paper describes the general method and the procedures which have been found useful in the application of the method to the most commonly encountered situations. Illustrating the procedures are examples of the applications to specific drug systems.

ONE OF the most seldom used, and yet potentially powerful methods for describing some pharmaceutical systems is the method of quantitative X-ray diffraction. Of the numerous advantages of this method, the most striking is the offer of absolute specificity combined with a high degree of accuracy. This feature, which is particularly desirable for drug systems, is unique. Further, the method can often be applied directly to complex mixtures such as final drug formulations without the need for separations, is usually rapid, and does not necessarily require a knowledge of all of the ingredients of an unknown mixture. The method applies only to the determination of crystalline ingredients, but the system containing the ingredients may be in any physical state.

Some mention of the possibilities of quantitative diffraction was first made by Hull as early as 1919 (1), but the first work reported was by Clark and Reynolds on the analysis of mine dust in 1936 (2). This work, and other work following (3), was based upon microphotometric density measurements of X-ray film following exposure. This method of measuring the intensity of diffracted X-rays was highly inaccurate and it was not until the advent of the Geiger counter spectrometer (4) that truly quantitative diffraction became possible. Some early papers describing Geiger techniques were on the determination of quartz (5, 6) and heavy metal carbides (7).

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The mathematical relationships pertinent to quantitative diffraction analysis were derived and published in an important fundamental paper by Alexander and Klug appearing in 1948 (8). This paper described conditions under which standard curves alone could be used, and under which standard curves based on internal standards were required, depending upon absorption effects. Relationships permitting, in certain instances, quantitative analysis of differential absorption systems without the use of internal standards were described in 1953 (9). In 1958, Copeland and Bragg (10) described special conditions under which calibration curves could be sometimes eliminated and multiple components determined.

All of the above papers refer to investigations of inorganic systems. Published reports on quantitative diffraction applications to organic systems are almost nonexistent. A notable exception is the application to the determination of sodium penicillin G, by Christ, Barnes, and Williams (11).

It is the purpose of this paper to describe the application of quantitative diffraction methods to typical pharmaceutical systems. In making this application, several situations may be encountered and each situation requires a somewhat different procedure. The procedures found useful for each of the commonly encountered situations are described and illustrated by specific examples.

THEORY

The feasibility of quantitative X-ray diffraction stems from the fact that the intensity of a diffracted beam of X-rays is a function of the amount of

diffracting material. The linearity of this response, for a fixed set of experimental conditions, depends upon the difference in the amount of absorption of X-ray energy between the compound of interest and its surrounding matrix. The absorption of any material depends upon the mass absorption coefficients of its constitutive atoms, which in turn are generally a function of atomic number for a given wavelength of radiant energy. Thus, as a compound differs chemically from its matrix, so will it differ in absorption for the radiant energy. The result of a large difference is a very nonlinear relationship between the amount of diffracting material and the diffracted intensity.

It is significant that in inorganic systems the probability of wide variation in X-ray absorption between the compound of interest and its surrounding matrix is high, whereas with organic systems the variation is usually low. This feature of organic systems results in an advantageous gain in linearity of response and allows, in many instances, the use of a simple calibration curve for the quantitative analysis of complex organic systems.

The diffraction of X-rays by a crystal is a well-known phenomenon. It occurs because the wavelengths of X-rays are of the same order of magnitude as the spacings between planes of crystalline lattices. A fundamental relationship, the Bragg equation, is $N\lambda = 2d \sin \theta$. A monochromatic X-ray beam (wavelength λ) is diffracted by a set of parallel, equally spaced planes (spacing d) within a crystal. Diffraction occurs only in precise directions (the angle θ) with respect to the planes, for each of several orders of diffraction (N).

A single crystal has a number of sets of parallel planes, oriented differently from one another, each set having a constant d spacing between its parallel planes. Diffraction from any single set of parallel planes is possible when the crystal is properly oriented with respect to an X-ray beam. If a sample consists not of a single crystal but of a large number of small crystals packed together with random orientation, all possible orientations of single crystals will be presented to a fixed X-ray beam. Such an arrangement is found in the goniometer shown in Fig. 1 where S represents an edge view of a shallow tray containing a finely powdered crystalline sample with a planar surface. A proportional counter C rotates about S at an angle of 2θ, while the sample turns at θ, with respect to the fixed X-ray source. Whenever a crystal in the randomly packed powder sample falls into such a position that the Bragg equation is satisfied, diffraction will occur and the position (2θ) and intensity of the diffracted ray is measured by the scanning counter.

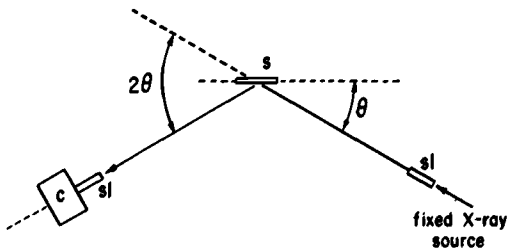

Fig. 1.—The sample (s) rotates through θ while the counter (c) scans through 2θ. An edge view of the planar-surface sample is shown. X-ray beam slits (sl) define the beam parallel to the sample surface.

Modern instrumentation permits this operation to be accomplished automatically. While the proportional counter is driven in its scanning operation at constant velocity, the sample is rotated at half this angular velocity, and a synchronized strip-chart recorder plots the diffracted intensity as a function of the angle, 2θ. For semiquantitative studies, strip-charts can be used directly but for truly quantitative work, scaler and timer circuits are employed, permitting the more accurate determination of the intensities of the diffracted rays.

When diffraction occurs at a given 2θ value, it does so because a sufficient number of crystals have the same set of planes, whose d spacings correspond to the 2θ value, properly oriented with respect to the X-ray beam. The intensity of the diffracted ray is a function of the amount of material so oriented. If truly random orientation is assured,[1] and except for absorption effects, the diffracted intensity becomes proportional to what may be termed the specific lattice volume. It is highly significant that when the intensity of a single diffraction peak is measured at a fixed 2θ value, both additive and constitutive effects are being measured. This unique fact is the basis for the specificity of quantitative diffraction analysis.

EXPERIMENTAL

For the experimental work reported, a General Electric XRD-5 unit and copper $K\alpha$ radiation were employed. The unit was equipped with a decade scaler and timer circuit, proportional counter, 1° beam slit, and 0.2° detector slit.

Simple Calibration Curves.—For the preparation of the standard curve for the determination of pamoic acid in a matrix of the pamoic acid salt of a basic antibiotic, use was made of synthetic mixtures containing known concentrations of pamoic acid in the antibiotic salt. Following thorough mixing and grinding in a mortar, each sample was poured into a standard 2-in. sample tray, which required about 200 mg. of material. In order to assure random crystal orientation, the excess powder was carefully removed by use of the edge of a glass microscope slide, and the sample surface finally packed slightly by use of the surface of a rough, low-grade blotting paper. This procedure eliminates large errors due to preferred orientation of the crystals.

The diffraction peak intensity of each sample was determined in the following manner. After placing the sample in the diffractometer, the goniometer was set for 26.25° 2θ and the instrument set to record the time required for the accumulation of 20,000 counts. This operation was followed by a setting of the goniometer at 26.80° 2θ and a second recording of the time required to accumulate 20,000 counts. The diffraction intensity at 26.25° (peak intensity) in counts-per-second was calculated, and the intensity at 26.80° (background intensity) in counts-per-second was also calculated. Since the statistical accuracy of a counting process is a function of the

[1] In all quantitative diffraction studies the effects of preferred orientation of the crystals must be minimized. Simple grinding, followed by careful packing in the sample tray, usually accomplishes this for organic compounds. In instances where an extreme of one crystal habit is represented, the advantageous use of 2% carbon black added to the mixture has been reported (11).

total number of counts taken, a preset count, rather than preset time, was used to determine the intensities in counts-per-second. This procedure allows all values, when compared later, to be of the same statistical accuracy.

The background intensity was subtracted from the peak intensity and the resulting value for each of the standard samples representing synthetic mixtures was plotted *vs.* the known concentration.

The calibration curve for the determination of novobiocin in mixtures of tetracycline and amorphous suspending agent was prepared from aqueous suspensions containing known amounts of novobiocin in mixtures of these ingredients. Solids were separated from suspension by centrifugation and air dried.

Use of an Internal Standard and Integrated Diffraction Peak Areas.—A method for the determination of a crystalline sulfonamide in aqueous suspension illustrates the use of integrated peak areas and an internal standard.

In this method, exactly 3.00 Gm. of powdered $CaSO_4 \cdot 2H_2O$, the internal standard, was added to 50.0 ml. of the sulfonamide suspension. As the suspension was too viscous to filter and too stable to separate with reasonable centrifuge times, it was diluted 1:4 with water which was freshly saturated with the pure sulfonamide. Centrifugation and decantation was followed by a second suspension in the dilution medium. A second centrifugation and decantation step freed the total solids from all water-soluble materials. The sulfonamide itself was virtually insoluble. The dried residue was mixed by grinding and packed into a standard diffraction sample tray. A portion of the diffraction pattern of the mixed solids from a 5% suspension of the sulfonamide is shown in Fig. 2. The doublet at 10° is from the sulfonamide diffraction, and the peak at 11.5° is from the internal standard.

The integrated intensity values were determined by the following procedure. With the scaler set for a preset time of 100 sec., the counting rate at 9° was determined in counts-per-second. This was repeated for positions at 11° and 13°. Diagrammatically these three values of background counting rates are represented in Fig. 2 by the lengths of the lines *AF*, *BE*, and *CD*.

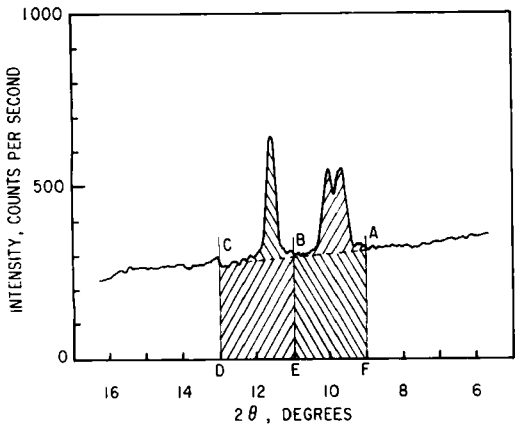

Fig. 2.—Partial diffraction pattern showing sulfonamide peaks (doublet at 10°) and internal standard peak (11½°).

With the goniometer set at somewhat less than 9°, an automatic scan was begun at 2° per minute. As the scan reached 9°, the counting switch was turned on, simultaneously starting the scaler and timer. As the scan crossed 11°, this switch was turned off. The total count and total elapsed time (approximately 60 sec.) were recorded. The elapsed time (in seconds) is represented by *EF* on the diagram and when multiplied by the average of background counting rates measured at 9° and 11° (in counts-per-second) gives the area of background under the the sulfonamide curve (*ABEF*) expressed in total count. This value was subtracted from the total count accumulated in the scan operation. The difference was the area of only the sulfonamide diffraction peak, expressed in total count. The operation was repeated between 11° and 13° to determine the area of the internal standard peak. The ratio of sulfonamide peak area to standard peak area was then computed.

RESULTS AND DISCUSSION

Use of Simple Calibration Curves.—No errors due to changing absorption effects occur with simple, two-component mixtures, or with multicomponent mixtures whose composition, except for the component being analyzed, remains constant. The determination of a crystalline ingredient in such systems may be based on calibration curves prepared from synthetic mixtures. An example is the determination of pamoic acid in a matrix of the pamoic acid salt of a basic antibiotic. The method was found useful in testing for completeness of the salt-forming reaction, and particularly in support of the long-term stability studies of a formulation of the antibiotic salt.

The diffraction pattern of pamoic acid is shown in Fig. 3. Although major diffraction peaks occur at 2Θ values of 9.50°, 11.20°, 15.60°, and 19.40°, all of these peaks are at least partially obscured when superposed on the pattern of the matrix materials. For this reason the peak at 26.25° 2Θ was chosen as a basis for the determination.[2] The results of a plot of intensity differences, peak minus background, for several known concentrations is shown in Fig. 4. The linearity shows the direct applicability to simple systems. The total counting time for each point was approximately 3 min.

Since organic molecules are large, organic crystals have large unit cells. Most interplanar spacings are, therefore, large. Such spacings give rise to diffraction peaks at small values of 2Θ which is the region of maximum response to scattering of the X-rays by the powdered sample. Thus it sometimes happens that a diffraction peak of interest occurs in an area of maximum background. This is particularly true when a sample contains a large amount of amorphous or scattering material.

Such a situation is illustrated by Fig. 5 which shows the superposition of diffraction peaks of novobiocin and tetracycline on a steep background slope. This diffraction pattern was measured using

[2] One has a choice of several possibilities upon which to base a proposed analysis. If the matrix is not obtainable in pure form, and there is doubt as to the freedom from interference of diffraction peaks, two simultaneous methods may be developed, each using a separate peak. Freedom from interfering peaks is assured when results of the determinations agree.

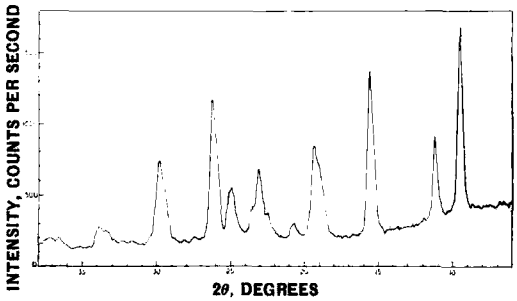

Fig. 3.—The pamoic acid diffraction pattern.

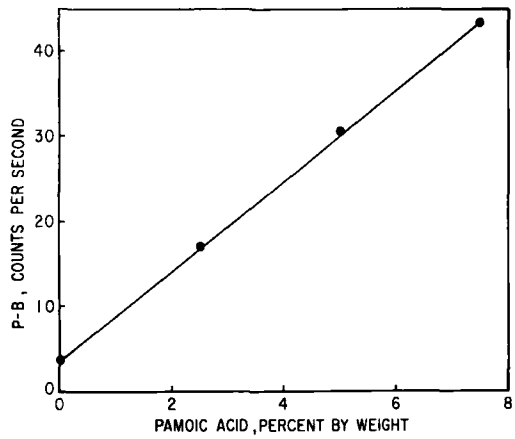

Fig. 4.—Intensity difference, peak minus background, as a function of pamoic acid concentration.

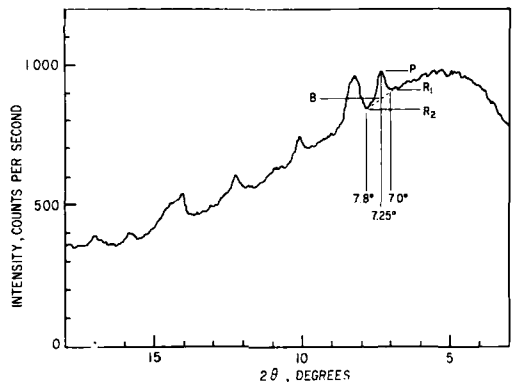

Fig. 5.—Partial diffraction pattern of an antibiotic mixture showing novobiocin peak at 7.25°.

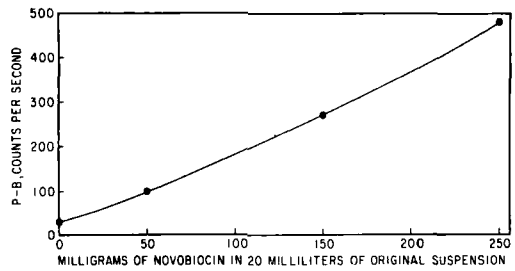

Fig. 6.—Intensity difference, peak minus background, as a function of novobiocin concentration.

the solids removed by centrifugation from a diluted aqueous suspension of calcium novobiocin, tetracycline, and an added known amount of crystalline novobiocin acid as a standard.

The diffraction peak at 7.25° 2θ (Fig. 5) is due to crystalline novobiocin acid. Its intensity is not influenced by the other peaks, which are due to tetracycline, but it is influenced by the unusually high background caused by the large percentage of amorphous suspending agent which was concentrated by the centrifugation.

A large part of the apparent novobiocin peak intensity is actually due to the background intensity. The true net peak intensity, upon which accuracy is based, is smaller. Moreover, reproducibility of the background intensity at the base of the peak is poor due to the steepness of the background slope.

Under these conditions it has been found advantageous to determine the net peak height by a different procedure in order to maintain accuracy. This procedure resulted in a close approach to linearity of response when the net peak intensity was plotted against concentration of crystalline novobiocin, for a series of prepared standard samples.[3] Using the scaler and synchronous timer circuits, the intensity P was measured at 7.25° 2θ (Fig. 4). The intensities at R_1 and R_2 were measured at 2θ values of 7.00° and 7.80°. The background B was calculated by interpolation

$$B = R_2 + \frac{7.80 - 7.25}{7.80 - 7.00}(R_1 - R_2) = R_2 + 0.688(R_1 - R_2)$$

The net peak height (P-B) was then calculated for each sample. These values were plotted against concentration of crystalline novobiocin. The results are shown in Fig. 6.

If all variables such as instrumental drift and the density of sample packing could be held constant, the background correction could be eliminated. Its use, however, permits day-to-day validity of the standard curve with good accuracy.

In the two previous examples, peak intensities were corrected by subtracting background intensities. It is just as valid to use a peak-to-background intensity ratio in place of the arithmetical difference. Moreover, the choice of position for the background measurement is not limited, an obvious advantage in instances of multiple peak overlapping. In the formation of a standard curve for the determination of mucic acid in mixtures of mucic acid and tetracycline,[3] peak-to-background ratios were used and the 2θ position for the background determination (29.00°) was necessarily some distance removed from the mucic acid peak of choice (19.48°). The resulting plot of intensity ratios *vs.* known concentration over the range to which interest was limited is shown in Fig. 7.

Use of an Internal Standard.—The most universally applicable method for quantitative diffraction involves the use of an internal standard. This method is free from all matrix effects as well as errors due to variations in sample packing density and variations in instrumental conditions. It is ideal for drug systems containing unknown mixtures, such as possible degradation products.

[3] The author is grateful to Drs. L. C. Schroeter and W. Morozowich, of The Upjohn Co., who prepared the samples for these standard curves.

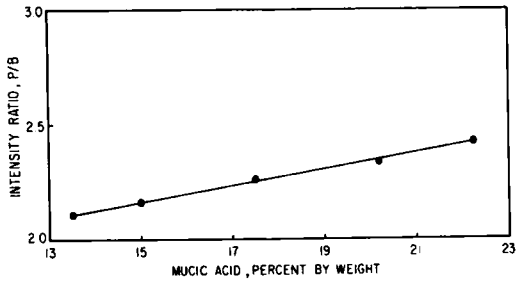

Fig. 7.—Intensity ratio, peak to background, as a function of per cent mucic acid in tetracycline.

The procedure involves examination of the diffraction patterns of the material to be assayed, and of the matrix in which it is found, and selecting a peak produced by the material which is free of interference from neighboring peaks. A suitable inert crystalline material, the internal standard, must then be found which has a peak in a clear region with respect to the system to be analyzed.

When a suitable internal standard has been found, a standard curve is prepared. Samples for the standard curve contain known but different concentrations of the compound to be analyzed and a fixed concentration of the internal standard. The proper concentration of internal standard to establish for use in all samples is one which is found to give a diffraction peak about equal in intensity to that of the material to be analyzed when the latter is present in the usually expected concentration.

The X-ray procedure then consists simply of determining the ratio of intensities of the diffraction peaks of the unknown and the internal standard, without regard for the background. The ratio is linearly proportional to the concentration of the unknown. Typical results of the use of an internal standard are shown in an example to follow.

Finding a suitable internal standard for each new system is often a problem. An ideal internal standard has several attributes: It must not have a diffraction peak which is obscured by matrix peaks, nor which will interfere with that of the material to be analyzed; the internal standard peak should be near the usable peak of the analyzed ingredient; it should be of high crystal symmetry, preferably isometric, so that strong but few diffraction peaks are produced and preferred orientation effects will be minimized by the more equant crystals; because of absorption effects, it should contain only elements of low atomic number; it should have a density not too far removed from those of the system ingredients for aid in maintaining homogeneity in mixing; and it should be chemically stable in the presence of the system. No one compound can qualify universally on all counts, but in addition to the compound used in an example to follow later, a material which has been found to have many of the above attributes is hexamethylenetetramine, one of the few organic compounds of isometric (cubic) symmetry. Another compound, beryllium acetate, has been proposed as an ideal standard since it meets most of the above specifications. Its extreme toxicity, however, would tend to disqualify it for use in pharmaceutical areas.

It is worth mentioning that in instances in which the system is of many variable components, and would therefore require an internal standard, but for which no suitable standard can be found, an alternative procedure exists. This consists of using known additions of the ingredient to be analyzed to the unknown system, plotting the peak intensity values as a function of the amount added, and extrapolating the curve. The intercept value gives a measure of the original amount of ingredient present.

Use of Integrated Diffraction Peak Areas.—Some organic compounds, when produced in successive batches over a period of time, tend to vary in the degree of crystallinity possessed at any one time. A decrease in the degree of crystallinity of a compound is accompanied by a drop in the apparent diffraction peak intensity as measured by the peak height. Such a drop in peak height, however, is accompanied by a peak broadening. It is significant that the area of the diffraction peak is relatively constant for a wide range of crystallinity. It is, therefore, sometimes of value to base a diffraction assay on peak areas rather than on peak heights. Further, the use of peak areas is advantageous when the particle size of the crystalline ingredient is very small due, again, to line broadening and a significant drop in peak height. Measurable line broadening occurs in the particle size range below 0.2 μ. Finally, the peak area method offers an advantage in being free from errors due to apparent shifts in peak maxima.

A standard curve for an assay based on the use of an internal standard and on integrated peak areas is always linear and intersects the origin.

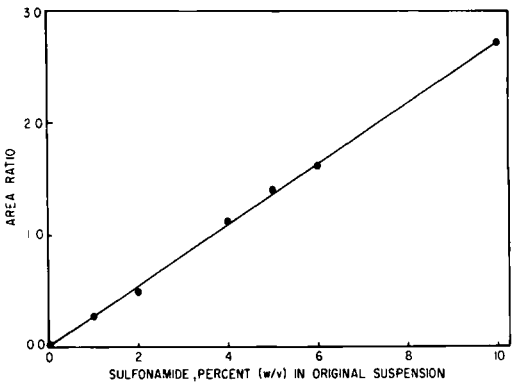

Fig. 8.—Ratio of peak areas, sulfonamide to internal standard, as a function of sulfonamide concentration.

A method for the determination of a sulfonamide in aqueous suspension illustrates the use of an internal standard, as well as integrated diffraction peak areas. Figure 8 shows a least squares plot of values for the ratios of sulfonamide peak areas to internal standard peak areas as a function of sulfonamide concentration. Following sample preparation, X-ray analysis required about 15 min. per sample. The values indicate an accuracy of $\pm 0.15\%$ of the amount present. The method is specific for the intact sulfonamide molecule in the crystalline state and, therefore, sensitive to any product degradation.

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Dissolution Rate Studies I

Continuous Recording Technique for Following Rapid Reactions in Solution

By PAUL J. NIEBERGALL† and JERE E. GOYAN

An automatic recording apparatus was developed to follow the process of dissolution. Results were obtained as a recording of per cent transmittance *vs.* time. The recorder was equipped with various speed chart drive motors that permitted readings to be taken from the graph at intervals down to 0.375 sec. Several experiments were performed to demonstrate the applicability of this apparatus to the determination of dissolution rates, and constants obtained agreed well with those obtained by the usual method of removing samples with a pipet.

THE FIRST quantitative study of the dissolution process was made by Noyes and Whitney in 1897 (1). Since that time numerous workers have evaluated the effect of different variables on the dissolution rate constant. The majority of these studies have been done using a single large tablet or disk. In a few instances, particularly in attempts to obtain a measure of mixing efficiency, multiparticulate systems have been used.

Initial dissolution rates in multiparticulate systems are rapid, and previous studies have been handicapped by the lack of an accurate method for sampling the solution in the early stages of the process. Therefore, the object of this study was to develop an automatic recording technique which would furnish continuous data and obviate the need for hand sampling in studies of rapid reactions.

THEORY

Calculation of Dissolution Rate Constants.—Hixson and Crowell (2) derived an equation for the dissolution of a single particle in which the surface area was allowed to change with time, using the following form of the Noyes-Whitney equation

$$V\frac{dw}{dt} = -KS(w_s - w_o + w) \quad \text{(Eq. 1)}$$

in which w is the weight of the particle at time t, K is a rate constant, S is the surface area of the particle, w_s is the weight of solid needed to saturate the volume, V, of solvent at a given temperature, and w_o is the initial weight of the particle. Using the property of geometrically similar solids, Hixson and Crowell replaced the surface area by

$$S = kw^{2/3} \quad \text{(Eq. 2)}$$

in which k is a constant containing the shape factor and the density of the particle.[1]

The basic equation of Hixson and Crowell has been extended for use in multiparticulate systems by assuming a system of N equal-sized particles. The total surface area, A, would then be equal to $N \times S$ or

$$A = kNw^{2/3} \quad \text{(Eq. 3)}$$

The total weight, W, for a sample of N equal sized particles would be

$$W = Nw \quad \text{(Eq. 4)}$$

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† Present address: Philadelphia College of Pharmacy and Science, Philadelphia, Pa.

[1] The particles used in this study had a height, h, greater than the diameter, d, which may result in a change in the h/d ratio during the process of dissolution, and the Hixson-Crowell equations would theoretically not be applicable. However, the salicylamide crystals were followed for a total change in weight which was felt to represent a negligible change in the h/d ratio. The benzoic acid crystals were followed to a much greater change in weight. However, the Hixson-Crowell equations did yield straight lines, and it was felt that the apparent rate constants obtained from these equations would be suitable for use in a comparison study of this nature. [See also the paper by Blumberg, *J. Phys. Chem.*, **63**, 1129(1959).]

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Substitution of this into Eq. 3, with the condition that the number of particles remains constant, gives

$$A = k_1 W^{2/3} \quad \text{(Eq. 5)}$$

This result, when substituted into Eq. 1, with the weight of a single particle replaced by the total weight of N particles gives

$$V \frac{dW}{dt} = -K_1 W^{2/3} (W_s - W_o + W) \quad \text{(Eq. 6)}$$

Two sets of conditions can be utilized in order to simplify this equation. When the amount of sample used is equal to the amount needed to saturate the solution, W_s equal to W_o, the equation becomes

$$V \frac{dW}{dt} = -K_1 W^{5/3} \quad \text{(Eq. 7)}$$

which upon integration under the condition that W is equal to W_o at time zero, gives

$$W^{-2/3} - W_o^{-2/3} = K_2 t \quad \text{(Eq. 8)}$$

in which K_2 now contains V.

When the amount of solute needed to saturate a given volume of solvent is much greater than the concentration of material in solution, $C_s \gg C$, the equation becomes

$$\frac{dW}{dt} = -K_3 W^{2/3} \quad \text{(Eq. 9)}$$

in which K_3 contains V and W_s. This equation, when integrated under the condition that W is equal to W_o at time zero, gives

$$W_o^{1/3} - W^{1/3} = K_4 t \quad \text{(Eq. 10)}$$

where K_4 is equal to $K_3/3$.

EXPERIMENTAL

Apparatus.—A model 5800 Beckman energy recording adapter was attached to a Beckman model DU spectrophotometer. The adapter leads were connected to a Varian Associates model G-10 potentiometric recorder. A tube bent into a U shape was made from silica glass, 5-mm. internal diameter. This was fitted into two holes drilled into a cell-compartment cover for the spectrophotometer and the protruding ends painted with black paint. The solution could then be passed through the tube and a continuous tracing of per cent transmittance *vs.* time obtained.

The remainder of the system consisted of a 1-L. beaker equipped with four baffles, a Cenco variable-speed stirrer and a Gordon-Rupp model 1612 centrifugal pump. The width of the baffles represented 10% of the beaker diameter; they were set about 1/8 in. out from the wall and 1/8 in. up from the bottom to eliminate dead pockets. The impeller was a four-blade turbine, 2 1/2 in. in diameter.

The beaker was placed in a constant temperature water bath and the impeller centered by means of appropriately placed markings. A glass filter stick was clamped into the beaker and the open end connected with rubber tubing to one end of the U tube. The other end of the U tube was connected with rubber tubing to the pump and a tube led from the pump back to the beaker.

Assay Procedure.—In this system, the solution coming from the beaker is assayed directly without dilution. Dissolution rates in multiparticulate systems are rapid, and for materials having high absorptivities, concentrations at which large deviations from the Beer-Lambert law occur are reached rapidly. This can be circumvented in some instances by assaying at absorption minima.

A series of dilutions was made for the material to be assayed. The apparatus was assembled as described above and the recorder was balanced at 0% transmittance with the shutter of the spectrophotometer closed. The pump was started and the recorder was balanced at 100% transmittance with the solvent flowing through the system and the shutter open, thus blanking out the absorbance due to the solvent. When the 100% transmittance base line had held steady for 2 minutes, the rubber tubing was clamped shut, and the glass tube cleaned and placed into one of the dilutions. The tubing was released and the solution allowed to flow through the apparatus until a steady transmittance tracing was obtained. The rubber tubing was again clamped and the glass tube placed into the next dilution. When all of the dilutions had been recorded, pure solvent was again passed through the system. If the base line did not level off at 100% transmittance, the calibration was repeated.

The values for per cent transmittance were converted into units of absorbance and plotted in the usual manner to obtain a Beer-Lambert line. The slopes of the resulting lines were calculated using the method of least squares and were used in the usual manner to calculate concentrations. Results for benzoic acid U.S.P. and salicylamide C.P. appear in Table I.

Table I.—Absorbance-Concentration Relationships of Benzoic Acid and Salicylamide in Water

Benzoic Acid		Salicylamide	
Concn., mg./100 ml.	Absorbance 259.5 mμ	Concn., mg./100 ml.	Absorbance 263 mμ
1.00	0.035	1.00	0.015
2.00	0.049	1.50	0.021
5.00	0.131	2.00	0.027
10.00	0.256	2.50	0.034
15.00	0.380	5.00	0.068
20.00	0.527	6.00	0.082
25.00	0.664	7.50	0.102
50.00	1.301	9.00	0.125
60.00	1.569	10.00	0.137
75.00	2.000	...	...
Absorptivity[a] S. D. 0.0002	0.0264	Absorptivity[a] S. D. 0.0001	0.0137

[a] Calculated by least squares as the absorbance of a 0.001% solution.

Dissolution with Return Flow to the Beaker.—In order to determine any systematic error due to the apparatus, the rate constant for the dissolution of salicylamide in water was determined using both the apparatus and the conventional method of sampling.

The salicylamide was carefully screened through a 20-mesh sieve onto a 30-mesh sieve. The particles retained on the latter were used in the study. The solubility of the salicylamide was found to be 2.44 Gm./L. at 25°.

The dissolution apparatus was assembled as described previously, and the total volume of the

glass filter tube, rubber tubing, U tube, and pump was determined by filling the system with water. This was repeated five times with the average being 55 ml. This volume of water was added to 750 ml. of distilled water in the beaker, and the system filled by siphoning. The pump was started with the water being circulated back into the beaker.

The recorder was balanced at 0% and 100% transmittance. When the contents of the beaker had reached temperature equilibrium, the end of the rubber tubing through which the water was passing into the beaker was placed into a 100-ml. graduated cylinder. An accurately weighed sample of salicylamide was simultaneously poured into the beaker. When the volume of liquid needed to fill the system (55 ml.) had been removed, the end of the rubber tubing was again placed so the solution would be returned to the beaker keeping the volume constant at 750 ml. It was felt that by this time (15 sec.) the concentration of the solution in the beaker was great enough that the dilution due to the returning solution would be negligible. The total time for the run was 30 sec. The stirring rate was 500 r.p.m. and the flow rate 350 ml. per minute.

The amount of salicylamide used was just sufficient to saturate the 750 ml. of water; Eq. 8 was applicable. Therefore, $W^{-2/3} - W_0^{-2/3}$ was plotted against time, resulting in a straight line which is shown in Fig. 1. The positive intercept represents a lag of 1.2 sec. This lag is not a factor of circulation time since time zero was taken as being the instant before the recording pen started to move. This lag will be discussed further in a future publication. The rate constant was evaluated using the method of least squares on the data shown in Table II.

TABLE II.—DISSOLUTION RATE OF 20/30-MESH SALICYLAMIDE CRYSTALS IN WATER AT 25° USING THE AUTOMATIC RECORDING APPARATUS

Time, sec.	Concn., mg./100 ml.	Weight Remaining (W), mg.	$W^{-2/3} \times 10^3$
0.00[a]	0	1830	6.733
3.75	2	1810	6.759
7.50	5	1790	6.838
11.25	9	1770	6.891
15.00	12	1735	6.970
18.75	15	1710	7.022
22.50	18	1690	7.101
26.25	21	1680	7.127
30.00	24	1655	7.206
33.75	27	1640	7.259
37.50	30	1610	7.311
41.25	32	1595	7.364
45.00	34	1585	7.443

$K_1 = 1.79 \times 10^{-2}$ mg.$^{-2/3}$ sec.$^{-1}$ ml.

[a] Time zero point omitted in calculation of the rate constant due to the time lag.

Circulation time should have no effect on the rate constant since it corresponds to frequency of sampling in the hand sampling case. This was verified by running the previous experiment at two other flow rates and calculating the rate constants. The results are given in Table III.

The effect of the dilution resulting from the return of the solution to the beaker could not be determined since the solution was thoroughly mixed while passing through the pump. If it had been serious, however, a break in the line shown in Fig. 1 would have been expected at the point representing the elapsed time of 15 sec.

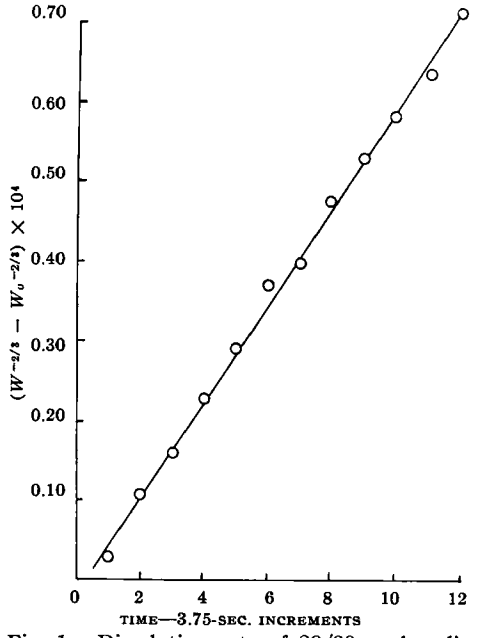

Fig. 1.—Dissolution rate of 20/30-mesh salicylamide in water at 25° using the automatic recording apparatus.

TABLE III. DISSOLUTION RATE CONSTANTS OF 20/30-MESH SALICYLAMIDE CRYSTALS WITH VARYING CIRCULATION TIMES

Flow Rate, ml./min.	Rate Constant (K_1), mg.$^{-2/3}$ sec.$^{-1}$ ml.
350	1.79×10^{-2}
150	1.81×10^{-2}
60	1.80×10^{-2}

The beaker was cleaned, filled with 750 ml. of distilled water, placed in the water bath, and stirred until temperature equilibrium had been reached. The same weight of salicylamide as used in the previous run was added and an electric timer started. Samples of 5 ml. were removed at intervals and assayed spectrophotometrically. The results are shown in Table IV and plotted in Fig. 2.

The values for the rate constants were compared

TABLE IV.—DISSOLUTION RATE OF 20/30-MESH SALICYLAMIDE CRYSTALS IN WATER AT 25° ON SAMPLES WITHDRAWN BY PIPET

Time, sec.	Concn., mg./100 ml.	Weight Remaining (W), mg.	$W^{-2/3} \times 10^3$
0	0	1830	6.696
15	13	1733	6.942
45	34	1575	7.392
75	52	1440	7.855
105	68	1317	8.338
125	80	1224	8.744
160	92	1130	9.223

$K_1 = 1.76 \times 10^{-2}$ mg.$^{-2/3}$ sec.$^{-1}$ ml.

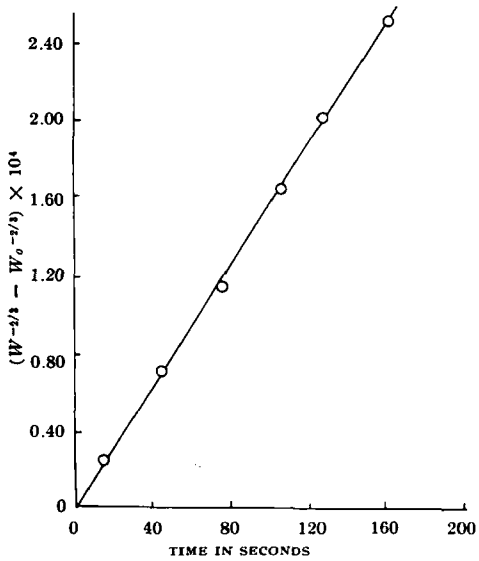

Fig. 2.—Dissolution rate of 20/30-mesh salicylamide in water at 25°. Samples withdrawn by pipet.

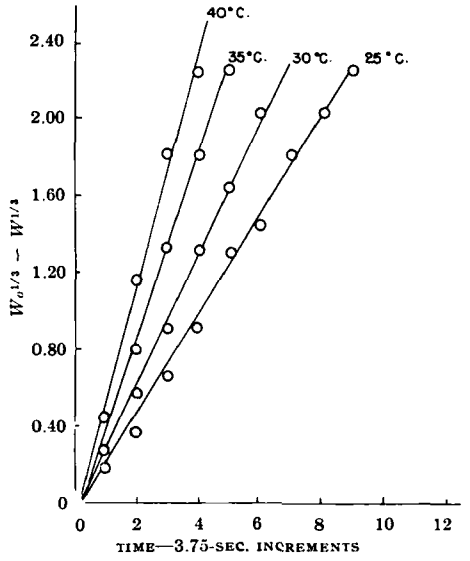

Fig. 3.—Dissolution rate of benzoic acid in water at various temperatures, using the automatic recording apparatus.

using the method described by Davies (3). The value of t was 0.945 with 16 degrees of freedom. The value of t necessary for the difference to be significant at the 95% level is 2.12. Therefore, there is no difference between the two values. It should also be noted that the standard deviation about the regression line was twice as large in the hand sampling case.

Dissolution Rate as a Function of Temperature.—One other experiment was performed to demonstrate the usefulness of the automatic recording apparatus. Reagent grade benzoic acid was screened to collect the 80/100-mesh fraction. The dissolution rate was obtained at four different temperatures using the automatic recording apparatus. In this study, however, the conditions met the requirements for the use of Eq. 10, C_s being much greater than C. The results are plotted in Fig. 3, and the rate constant K_4 was evaluated from the data shown in Table V.

TABLE V.—DISSOLUTION RATE OF 80/100-MESH BENZOIC ACID CRYSTALS IN WATER AT VARYING TEMPERATURES[a]

Time, sec.	Concn., mg./100 ml.	Weight Remaining, mg.
	25°C.	
0.00	0	158
3.75	2	143
7.50	4	128
11.25	7	106
15.00	9	91
18.75	12	68
22.50	13	61
26.25	15	46
30.00	16	38
33.75	17	31
	30°C.	
0.00	0	158
3.75	2	143
7.50	6	113
11.25	9	91
15.00	12	68
18.75	14	53
22.50	16	38
	35°C.	
0.00	0	158
3.75	3	136
7.50	8	98
11.25	12	68
15.00	15	46
18.75	17	31
	40°C.	
0.00	0	158
3.75	5	121
7.50	11	76
11.25	15	46

[a] Time zero point omitted in calculation of the rate constants due to the time lag.

This constant contains W_s, the effect of which was canceled by dividing K_4 by the W_s values taken from Edwards' work (4), resulting in a new constant, K_5.

Values for log K_5 were plotted against $1/T$ as shown in Fig. 4. The energy of activation calculated from the data shown in Table VI was 5210 cal./mole. Edwards (4) reported a value of 5162 cal./mole for the dissolution of benzoic acid in water, using a method of sampling similar to the one used in the above study.

DISCUSSION

The good agreement between the dissolution rate constant obtained using the automatic recording technique and the conventional method of withdrawing samples with a pipet indicate that the recording technique is valid for following rapid reactions in solution.

Standard deviations were obtained on the

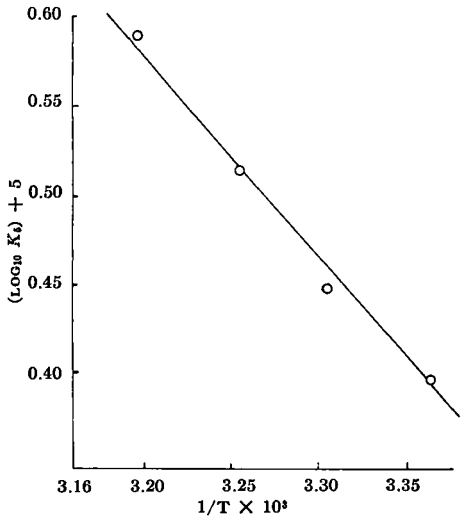

Fig. 4.—Arrhenius plot of the rate constants obtained for the dissolution of benzoic acid in water using the automatic recording apparatus.

values for the energy of dissolution as reported by Edwards' and those obtained in this study. The data from Edwards' paper give a standard deviation of 750 cal./mole, while the results obtained using the recording apparatus gave a standard deviation of 200 cal./mole. Thus, the apparatus appears to be particularly useful for working at elevated temperatures since it eliminates the problem of temperature change during the collection and subsequent handling of samples.

TABLE VI.—RATE CONSTANTS AS A FUNCTION OF TEMPERATURE FOR THE DISSOLUTION OF BENZOIC ACID IN WATER

Temp., °K.	K_4	Weight Needed for Saturation[a]	$K_5 \times 10^5$
298	0.07227	2565	2.818
303	0.09890	3075	3.216
308	0.13410	3623	3.701
313	0.18271	4163	4.389

[a] Taken from Edwards' data (4), and given as mg. needed to saturate 750 ml. of water.

One further advantage for the apparatus is that it can be cleaned and assembled for further use within 15 minutes after the completion of a run. This is a vast saving in time over that generally needed to clean volumetric glassware used in a kinetic study. This apparatus is therefore suggested for use in the study of rapid reactions in which one chemical species can be assayed spectrophotometrically with no interference from other species in solution.

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Investigation of the Mechanism of Urea-Induced Hemolysis

By DALE E. WURSTER and PAUL H. SHAPIRO†

The hemolysis of mammalian erythrocytes in concentrated urea solutions and in isotonic sodium chloride and dextrose solutions containing thirty per cent urea was investigated. Increased concentrations of sodium chloride, dextrose, and other osmotic agents inhibited the urea-induced hemolysis. The existence of urea–dextrose complexes in water, methanol, and methanol–water systems containing high dextrose concentrations were demonstrated.

DURING the last decade, Javid and Settlage, *et al.*, developed a remarkably effective method for reducing elevated intracranial and intraocular pressures. The method involves the intravenous administration of 30 per cent urea solutions (1, 2). In preliminary work with animals they observed that the administration of concentrated solutions caused a hemolysis which was detectable by the resulting hemoglobinuria. This would be expected to happen since the erythrocyte is permeable to urea, and thus the urea solution can exert no osmotic pressure. However, it was found that hemolysis also occurred when the urea was dissolved in an isotonic solution of sodium chloride or dextrose. In experimenting with the addition of various con-

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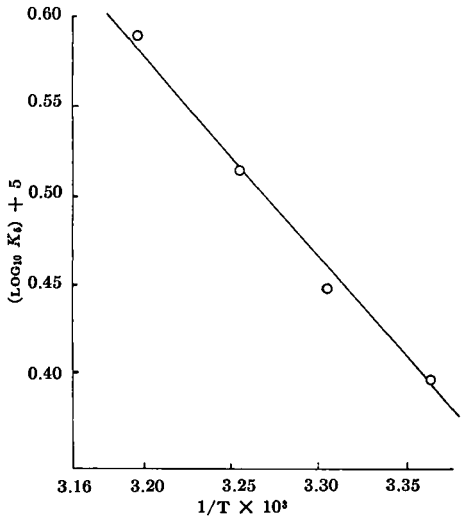

Fig. 4.—Arrhenius plot of the rate constants obtained for the dissolution of benzoic acid in water using the automatic recording apparatus.

values for the energy of dissolution as reported by Edwards' and those obtained in this study. The data from Edwards' paper give a standard deviation of 750 cal./mole, while the results obtained using the recording apparatus gave a standard deviation of 200 cal./mole. Thus, the apparatus appears to be particularly useful for working at elevated temperatures since it eliminates the problem of temperature change during the collection and subsequent handling of samples.

TABLE VI.—RATE CONSTANTS AS A FUNCTION OF TEMPERATURE FOR THE DISSOLUTION OF BENZOIC ACID IN WATER

Temp., °K.	K_4	Weight Needed for Saturation[a]	$K_5 \times 10^5$
298	0.07227	2565	2.818
303	0.09890	3075	3.216
308	0.13410	3623	3.701
313	0.18271	4163	4.389

[a] Taken from Edwards' data (4), and given as mg. needed to saturate 750 ml. of water.

One further advantage for the apparatus is that it can be cleaned and assembled for further use within 15 minutes after the completion of a run. This is a vast saving in time over that generally needed to clean volumetric glassware used in a kinetic study. This apparatus is therefore suggested for use in the study of rapid reactions in which one chemical species can be assayed spectrophotometrically with no interference from other species in solution.

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Investigation of the Mechanism of Urea-Induced Hemolysis

By DALE E. WURSTER and PAUL H. SHAPIRO†

The hemolysis of mammalian erythrocytes in concentrated urea solutions and in isotonic sodium chloride and dextrose solutions containing thirty per cent urea was investigated. Increased concentrations of sodium chloride, dextrose, and other osmotic agents inhibited the urea-induced hemolysis. The existence of urea–dextrose complexes in water, methanol, and methanol–water systems containing high dextrose concentrations were demonstrated.

DURING the last decade, Javid and Settlage, *et al.*, developed a remarkably effective method for reducing elevated intracranial and intraocular pressures. The method involves the intravenous administration of 30 per cent urea solutions (1, 2). In preliminary work with animals they observed that the administration of concentrated solutions caused a hemolysis which was detectable by the resulting hemoglobinuria. This would be expected to happen since the erythrocyte is permeable to urea, and thus the urea solution can exert no osmotic pressure. However, it was found that hemolysis also occurred when the urea was dissolved in an isotonic solution of sodium chloride or dextrose. In experimenting with the addition of various con-

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centrations and combinations of salts and sugars it was found that 10 per cent invert sugar was a suitable agent for preventing this hemolysis.

The purpose of this investigation was to obtain additional information on the mechanism of the urea-induced hemolysis. Equally important was a desire to study the effects produced by sugars in these systems and to determine in what manner they inhibited hemolysis. Since beef erythrocytes were used in this study, a quantitative transfer of the experimental data to a system containing human erythrocytes is not possible; however, it is reasonable to expect that the data would apply, qualitatively.

Hemolysis can be caused by two general methods, the lowering of the osmotic pressure of the medium in which the cells are suspended and by the direct action of a substance on the cell membrane. There are other means of causing hemolysis, but these methods are the most important.

In the early stages of the study it was suggested that urea might cause hemolysis by the latter method. Since the erythrocyte is permeable to urea, hemolysis occurs when erythrocytes are suspended in a solution containing any concentration of urea in water. Since hemolysis also occurred in a system containing 0.9 per cent sodium chloride plus 30 per cent urea, it seemed possible that the urea was attacking the cell membrane. Urea has previously been reported to complex with proteins and long chain fatty acids (3) and these substances are, of course, present in the cell membrane (4). Therefore, the possibility that such a complex was causing the disruption of the cell membrane was strongly considered. It was also possible that the sugars were adsorbed on the cell membrane and thereby protecting it from the urea or else forming a complex with the urea and thereby blocking its hemolytic action on the cell membrane. Several investigators have noted the inhibition of hemolysis by nonelectrolytes (5–9).

As the study progressed, however, there was reason to believe that urea caused hemolysis not by exerting any specific action on the cell membrane, but by the general action of a penetrating molecule. The study was then approached from this viewpoint.

EXPERIMENTAL

Source of the Blood.—The blood used in this study was obtained from beef cattle immediately after killing. The blood was collected in 125-ml. glass-stoppered Erlenmeyer flasks into which had previously been placed 0.25 ml. of heparin sodium injection U.S.P. After mixing, the flasks were immediately refrigerated.

Hemolysis Conditions.—The 100-ml. volumetric flasks containing 49 ml. of the particular solution were placed in a shaking apparatus in a 30° constant temperature bath. After allowing sufficient time for the solutions to reach 30°, 1 ml. of the whole blood was pipeted into each flask. The shaker was started and the flasks were maintained in the bath for 1 hour. Then, about 15 ml. of each suspension was poured into 15-ml. centrifuge tubes which were immediately centrifuged at 2000–2500 r.p.m. for about 15 minutes in a Servall type SP/X angle centrifuge.

Determination of Hemoglobin.—The analytical method for the determination of hemoglobin was a modification of that described by Finholt (10). Ten milliliters of the supernatant liquid from the centrifuge tubes was removed by pipet and placed in 25-ml. volumetric flasks. To these solutions was added 1 ml. of a 0.2% potassium cyanide–1% potassium ferricyanide solution. After waiting 15 minutes for the cyanomethemoglobin to form, the absorbance of the solutions was read at 545 mμ on a Cary model 11 recording spectrophotometer using 1-cm. silica cells.

Calculation of Per Cent Hemolysis.—The hemoglobin was assayed for the relative, not the absolute concentration. In each experiment one sample was included which contained 2% blood in water or in 30% urea, depending upon whether or not the other samples contained urea. The absorbance of this sample was taken as the 100% hemolysis reading. The per cent hemolysis of the samples was determined by dividing the absorbance of the sample by the 100% hemolysis reading.

Urea-Dextrose Complex in Methanol.—In the case of 100% methanol as the solvent, 2 Gm. of dextrose was placed in a series of 30-ml. vials along with varying amounts of urea and 20 ml. of methanol. The vials were placed in a shaker in a 30° constant temperature bath. After three days, 10 ml. was withdrawn from each vial and placed in tared containers. After the solvent was evaporated, the weight of dextrose was determined by subtracting the appropriate weight of urea. When 80 and 90% methanol were used as the solvent, 10 Gm. of dextrose was added and a 2-ml. aliquot was withdrawn.

Urea-Dextrose Complex in Water.—Varying amounts of urea were weighed into 4-dram vials. Into each vial was placed 3 Gm. of dextrose and 5 ml. of a saturated solution of dextrose. The vials were maintained with agitation in a 25° constant temperature bath for at least one week.

Upon removal from the bath, the entire contents of each vial was filtered through a 15-M sintered glass filter into a 100-ml. volumetric flask. Then the interior of the vial and the contents of the filter were washed repeatedly with 95% ethanol until the 100-ml. volumetric flask was filled. Three milliliters of this solution was diluted to 1,000 ml. with water. Two milliliters of this dilution was assayed for dextrose. In this case, the dextrose was determined according to the method of Nelson (11) and Somogyi's modification (12).

RESULTS AND DISCUSSION

Effect of 30 Per Cent Urea Plus Varying Sodium Chloride Concentration.—Since the erythrocyte is

TABLE I.—PER CENT HEMOLYSIS WITH 30% UREA AND VARYING SODIUM CHLORIDE CONCENTRATION

Age of Blood, Days	Per Cent Hemolysis — Sodium Chloride Concentration					
	0.7%	0.9%	1.1%	1.3%	1.5%	1.8%
0	100	100	74	24	24	14
1	100	100	74	15	15	19
2	100	97	61	12	12	10
3	100	92	43	4	4	4
4	100	100	97	16	16	19

TABLE II.—PER CENT HEMOLYSIS WITH 0.9% SODIUM CHLORIDE AND VARYING UREA CONCENTRATION

Age of Blood, Days	Per Cent Hemolysis — Urea Concentration					
	20%	22%	24%	26%	28%	30%
0	11	16	51	67	93	98
1	3	20	42	68	94	100
2	5	17	37	69	95	100
3	3	14	36	67	93	97
4	8	14	44	67	94	97

permeable to urea, if urea is the only dissolved substance in the suspension then hemolysis will occur regardless of the urea concentration. It was previously thought, however, that this permeability would not affect the cell adversely as long as the urea was dissolved in an isotonic solution of sodium chloride. Since hemolysis did occur under these conditions, it appeared possible that urea caused the hemolysis by attacking the cell membrane. However, it was found that when beef erythrocytes were suspended in medium containing 30% urea plus higher concentrations of sodium chloride, the per cent of hemolysis decreased with an increase in the sodium chloride concentration. The results of one such experiment are shown in Table I. Several of the earlier experiments were repeated on five consecutive days to determine what effects storing the blood in a refrigerator would have on the results.

On each day, the degree of hemolysis decreased as the sodium chloride concentration increased. As the sodium chloride concentration increased, increasing volumes of water necessarily left the erythrocyte. When the suspension medium contained 30% urea, a large volume of urea molecules diffused into the erythrocyte. When the sodium chloride concentration was low, the cell membrane was not able to accommodate the large increase in cellular content and it finally broke. Presumably, if the sodium chloride concentration was elevated sufficiently, a large enough quantity of water would leave the cell to enable the cell membrane to accommodate a large volume of urea without breaking.

For some of the intermediate concentrations of sodium chloride, the per cent hemolysis decreased daily for four days. This may be due to the constant leakage of potassium from the erythrocytes, as reported by Davson and Danielli (14). It is possible that the increased degree of hemolysis on the fifth day was due to some deterioration in the cell membrane.

Effect of Varying the Urea Concentration at a Constant Sodium Chloride Concentration.—The critical volume of the erythrocyte is about 1.6 times greater than the normal volume (15). If urea caused hemolysis as was described, there should be a minimum concentration below which no hemolysis should occur at any one concentration of sodium chloride. Table II shows the results of an experiment in which the urea concentration was varied, but the sodium chloride concentration was maintained constant at 0.9%.

The results indicated that when beef erythrocytes were suspended in 0.9% sodium chloride, the cells could accommodate approximately 20% urea before the critical volume was reached. As the concentration of urea was increased above 20 per cent, the degree of hemolysis increased rapidly.

Effect of Methylurea.—If urea caused hemolysis by the bulk of its molecules increasing the volume of the cell to the breaking point, then methylurea, having a greater molecular volume, should show a somewhat more pronounced effect. An experiment in which equal molar concentrations of the two compounds were compared showed that this was the case. The compilation of results from several experiments are given in Table III.

TABLE III.—A COMPARISON OF THE PER CENT HEMOLYSIS WITH UREA AND METHYLUREA

Molar Concentration	Per Cent Hemolysis	
	Urea	Methylurea
0.25	9	50
0.292	5	11
0.33	9	17
0.375	23	34
0.392	33	89
0.409	54	100
0.417	75	100

Effects of Dextrose.—It has been shown that urea-induced hemolysis can be inhibited by increasing the sodium chloride concentration in the medium. It was assumed that this effect could be produced by adding an osmotic equivalent of any nonpenetrating compound, but this assumption was not completely correct. Dextrose proved to be a far more effective agent in preventing hemolysis. Table IV shows a comparison of equal osmotic concentration of sodium chloride and dextrose.

It is interesting to note that the presence of urea affected the results only quantitatively. It was mentioned previously that erythrocytes are reported to have increased osmotic resistance when

TABLE IV.—COMPARISON OF HEMOLYSIS OF SODIUM CHLORIDE AND DEXTROSE WITH AND WITHOUT UREA

Concentration of Osmotic Agent		Per Cent Hemolysis	
		30% Urea	No Urea
Isotonic concn. 0.6	0.54% Sodium Chloride	100	71
	3.03% Dextrose	34	5
	0.585% Sodium Chloride	100	41
Isotonic concn. 0.65			
	3.28% Dextrose	13	2
	0.63% Sodium Chloride	100	21
Isotonic concn. 0.7			
	3.54% Dextrose	18	0

placed in a solution of a nonelectrolyte. These results with and without urea further indicate the similarity between osmotic hemolysis and urea-induced hemolysis.

The observation that erythrocytes behave abnormally when placed in a solution of a nonelectrolyte implies that it is the absence of electrolyte that causes the difference. To investigate this further, solutions were prepared which contained combinations of sodium chloride and dextrose such that the combination of the two compounds yielded a solution that was 0.6 isotonic. No urea was included. These results are given in Table V.

TABLE V.—PER CENT HEMOLYSIS WITH VARYING COMBINATIONS OF SODIUM CHLORIDE AND DEXTROSE

Concn. Sodium Chloride, %	Concn. Dextrose, %	Hemolysis, %
0.27	2.02	30
0.18	2.52	18
0.09	3.03	11
0	3.54	2

The increase in sodium chloride concentration from zero to 0.18 per cent caused a marked response even though this was only a 0.03 molar solution. This suggested that the dextrose did not affect the cell membrane directly, but acted only by allowing electrolyte to be excluded from the medium.

Effects of Other Sugars.—Dextrose was the primary sugar studied in this investigation. The results in Table VI show that hypotonic lactose and sucrose solutions cause an increased osmotic resistance similar to the effect produced by dextrose.

TABLE VI.—PER CENT HEMOLYSIS IN HYPOTONIC LACTOSE AND SUCROSE SOLUTIONS

Concentration of Osmotic Agent		Per Cent Hemolysis
Isotonic concn. 0.6	5.85% Lactose	7
	5.55% Sucrose	6
	6.34% Lactose	3
Isotonic concn. 0.65	6.01% Sucrose	3
	6.82% Lactose	2
Isotonic concn. 0.7	6.48% Sucrose	2

Blood in isotonic fructose (5.05%) plus 30% urea showed only 6% hemolysis. Thus, it is evident that all of these sugars gave very similar results. It is probable that all four sugars act simply by allowing electrolyte to be excluded from the medium.

Other Effects of Sugars.—On several occasions when blood was suspended in an isotonic solution of dextrose, lactose, sucrose, or inositol, within 15 minutes the red cells aggregated into large masses which immediately settled to the bottom of the flask. Upon the addition of very small quantities of one of several salts such as sodium chloride, sodium bromide, or potassium bromide, it was immediately possible to resuspend the red cells to form an apparently normal suspension. The addition of sufficient sodium chloride to give a concentration of only 0.18% was sufficient to bring about this reversal.

Effects of Other Electrolytes.—The only electrolyte reported on extensively thus far has been sodium chloride. Therefore, an attempt was made to compare the other electrolytes with sodium chloride to determine whether the erythrocyte reacted similarly to electrolytes in general. Table VII is a compilation of the results of several experiments on several different samples of blood.

The results showed that whenever the anion was divalent or trivalent, there was little or no hemolysis. There was a danger, of course, that in working with such a wide variety of salts in unbuffered solutions, that changes in the pH of the medium would become important. However, the results suggested that perhaps it was not the presence of

TABLE VII.—PER CENT HEMOLYSIS WITH HYPOTONIC CONCENTRATION OF VARIOUS SALTS

Salt	Concn., %[a]	Hemolysis, %
NaCl	0.54	80
KCl	0.741	76
NaI	1.42	94
KI	1.55	46
$NaC_2H_3O_3$	0.708	96
$NaNO_3$	0.816	62
KNO_3	0.972	76
$KClO_4$	1.13	93[b]
$CaCl_2 \cdot 2H_2O$	1.02	74
Calcium Lactate	2.70	79
$NaH_2PO_4 \cdot H_2O$	1.47	72
Na_2HPO_4 (anhyd.)	1.05	3
Na_2SO_4	0.986	4
K_2SO_4	1.27	2
$CuSO_4 \cdot 5H_2O$	4.11	0[c]
$MgSO_4$	3.78	2
Sodium Citrate	1.81	0
$Na_2B_4O_7 \cdot 10H_2O$	1.56	0
$Na_2S_2O_3$	1.79	0

[a] 0.6 of isotonic. [b] Turned brown in color. [c] Cupric ion reacted with the cyanide ferricyanide reagent. However, visual observation indicated little or no hemolysis.

electrolyte *per se* which determined whether or not the erythrocyte behaved normally, but that the anion was the important moiety.

Urea-Dextrose Complex.—It was mentioned previously that a urea-dextrose complex could be a contributing factor to the ability of dextrose to inhibit urea-induced hemolysis. Since the erythrocyte is impermeable to dextrose, such a complex would serve to reduce the effective concentration of urea. The problem was to demonstrate the existence of such a complex. The small size of both molecules combined with their very high water solubility and low solubility in solvents made it very difficult to detect a complex. Because of the small molecular size, it was not possible to use a semipermeable membrane to follow the complex formation. The high water solubility also made it very difficult to measure changes in concentration in two immiscible solvents as the complex formed. However, it was possible to demonstrate the existence of urea-dextrose complex in water, methanol, and water-methanol mixtures by using the solubility method. Unfortunately, it was necessary to use saturated solutions of dextrose to do so. It would have been much more desirable to use the same concentrations of dextrose that were used in the hemolysis studies.

In the case where the complex was studied in water, a further complication was provided by the large variation in the volume of the various samples caused by the amount of urea included. This large fluctuation in volume made it extremely difficult to determine the concentrations of urea and dextrose in the solutions. Since it was necessary to remove the vials from the bath for the dextrose determination, the complex in water was thermostated at 25° instead of 30°. The former temperature being closer to room temperature made it less likely that a temperature change would disturb the complex.

Figure 1 shows that as urea was added to a saturated solution of dextrose in water, the quantity of dextrose in solution increased. Since the precise concentrations of urea and dextrose were difficult to determine, it was not possible to calculate the equilibrium constant of the complex. The plateau of the curve was used to calculate the stoichiometric ratio of the complex (16). The ratio of urea to dextrose was probably in the neighborhood of

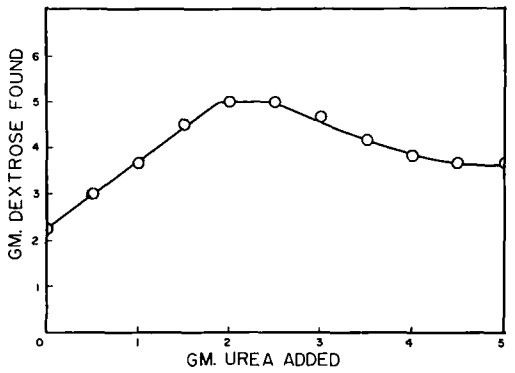

Fig. 1.—Phase diagram of dextrose–urea system in water at 25°.

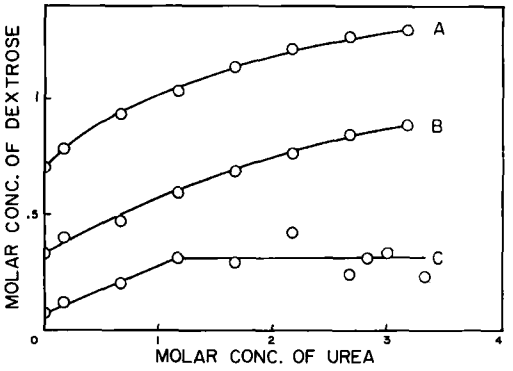

Fig. 2.—Phase diagrams of dextrose–urea systems in methanol–water and methanol at 30°. (Curve *A* is 80% methanol, curve *B* is 90% methanol, and curve *C* is 100% methanol.)

3:1 to 4:1. The uncertainty arose from the difficulty in ascertaining the exact length of the plateau.

The low solubility of dextrose in methanol made it easier to demonstrate the existence of a complex in this solvent. Figure 2 shows the results that were obtained with three concentrations of methanol. In curve A, 80% methanol was used as the solvent; curve B represents 90%; and pure methanol was employed as the solvent in curve C.

Thus, there was little doubt that under certain conditions a urea-dextrose complex did indeed exist. However, because of the aforementioned solubility problems, it was not possible to demonstrate the existence of this complex at low dextrose concentrations in water. If urea did complex with dextrose at low dextrose concentrations this would become a very important factor in the inhibition of urea-induced hemolysis.

SUMMARY

Various aspects of urea-induced hemolysis have been investigated. It was shown that high concentrations of urea cause hemolysis in the presence of isotonic concentrations of other compounds by a sufficient volume of urea molecules diffusing into the cell to cause it to rupture. Therefore, a sufficiently large concentration of any nonpenetrating compound in the medium will inhibit urea-induced hemolysis.

Experimental evidence was obtained which indicates that it is necessary for electrolyte to be present in the medium in order to maintain a normal suspension of the erythrocytes. There is also evidence that the anion may be the important ion of the electrolyte in maintaining the integrity of the cell.

The existence of a urea-dextrose complex has been demonstrated at high dextrose concentrations. If this complex forms at low dextrose concentrations it would be an important factor in the ability of dextrose to inhibit urea-induced hemolysis.

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Softening and Liquefaction Temperature of Suppositories

By IVO SETNIKAR and SERGIO FANTELLI

A method is described for testing the softening and liquefaction temperature of suppositories. The data obtained yield the maximum environmental temperature at which suppositories retain sufficient firmness for handling and for ordinary storage. The physical properties of 44 bases were investigated by this new method. The melting point was determined by the open capillary method. Of 27 fatty bases, only 8 present properties which are as satisfactory as or better than those of theobroma oil. Most of the 17 water-soluble bases investigated have very satisfactory firmness, but their melting point is above body temperature so that, for liquefaction, they must dissolve in water drawn from the rectal mucosa.

RECTAL suppositories, if well formulated, should melt, soften, or dissolve when introduced into the rectum and yet retain their firmness and shape in ordinary storage conditions.

Melting, softening, or dissolving may be tested in an apparatus which reproduces the physical conditions of the rectum (1). This apparatus, however, does not give indications as to the firmness of the suppositories. The following characteristics may be examined in order to test firmness.

Melting temperature.—Melting temperature may be determined (*a*) by the open capillary method for fatty substances (*cf.* U.S.P. XVI, p. 926, class II) or by the sealed capillary method (2). Only suppository bases can be tested and the results are influenced by the technique used in the preparation of the capillary (2). (*b*) The suppository may be put in an aqueous environment whose temperature is raised until the suppository melts (3–6). In these conditions only strictly water-insoluble suppositories can be tested. Suppositories containing water-soluble substances soften or dissolve in the aqueous environment irrespective of temperature. (*c*) The temperature of the air surrounding the suppository may be gradually raised until melting. This method was followed by Erbe (7) who used an apparatus described by Bogs (8).

Hardness—Malangeau (3) molded a cylinder with a diameter of 9 mm. of the same composition of the suppository and determined the temperature at which the cylinder collapsed under a weight of 500 Gm. (8 Gm./-mm.^{2}). Münzel (9) and other authors (2, 10, 11) used the penetrometric method described in U.S.P. XIV, p. 693. Büchi and Oesch (12) stated that a suppository, at a temperature of 22°, should not be deformed by a weight of 500 Gm.

The first two methods cannot be used directly on suppositories while the method of Büchi and Oesch can, though it gives only limited information on the maximal temperature at which a suppository still shows sufficient mechanical stability. We describe here a method for determining the softening and melting temperatures directly on suppositories.

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RECTAL suppositories, if well formulated, should melt, soften, or dissolve when introduced into the rectum and yet retain their firmness and shape in ordinary storage conditions.

Melting, softening, or dissolving may be tested in an apparatus which reproduces the physical conditions of the rectum (1). This apparatus, however, does not give indications as to the firmness of the suppositories. The following characteristics may be examined in order to test firmness.

Melting temperature.—Melting temperature may be determined (*a*) by the open capillary method for fatty substances (*cf.* U.S.P. XVI, p. 926, class II) or by the sealed capillary method (2). Only suppository bases can be tested and the results are influenced by the technique used in the preparation of the capillary (2). (*b*) The suppository may be put in an aqueous environment whose temperature is raised until the suppository melts (3–6). In these conditions only strictly water-insoluble suppositories can be tested. Suppositories containing water-soluble substances soften or dissolve in the aqueous environment irrespective of temperature. (*c*) The temperature of the air surrounding the suppository may be gradually raised until melting. This method was followed by Erbe (7) who used an apparatus described by Bogs (8).

Hardness—Malangeau (3) molded a cylinder with a diameter of 9 mm. of the same composition of the suppository and determined the temperature at which the cylinder collapsed under a weight of 500 Gm. (8 Gm./-mm.^{2}). Münzel (9) and other authors (2, 10, 11) used the penetrometric method described in U.S.P. XIV, p. 693. Büchi and Oesch (12) stated that a suppository, at a temperature of 22°, should not be deformed by a weight of 500 Gm.

The first two methods cannot be used directly on suppositories while the method of Büchi and Oesch can, though it gives only limited information on the maximal temperature at which a suppository still shows sufficient mechanical stability. We describe here a method for determining the softening and melting temperatures directly on suppositories.

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METHODS

The apparatus (Fig. 1) consists of a 200-mm. long glass tube with an internal diameter 1–3 mm. larger than the maximal diameter of the suppository to be tested. The tube, in the middle part, has a constriction with an internal diameter of 3 mm. This constriction supports the suppository. The tube is surrounded by a glass mantle fitted with two connections through which water circulates. The temperature of the circulating water can be read on a thermometer.

The suppository is introduced into the upper part of the tube as shown in Fig. 1. Then a 120-mm. long glass rod, which has a diameter 2 mm. smaller than the internal diameter of the tube, is placed on the suppository. A short thick-walled rubber tube is slipped over the glass rod in such a way that, when the rod rests on the suppository, there is a distance of 14 mm. between the lower end of the rubber and the upper end of the glass tube. The rubber supports a lead cylinder which moves freely on the glass rod. The lead cylinder and glass rod with rubber have a weight of 500 Gm. when the test is performed on suppositories of a diameter of 9–10 mm. For thinner suppositories, the weight should be about 7 Gm./mm.^{2}. When the suppository collapses, the lead cyclinder sinks 5 mm., then it is stopped by a support which prevents any further sinking of the lead. We define the temperature at which this happens as the softening temperature (*ST*).

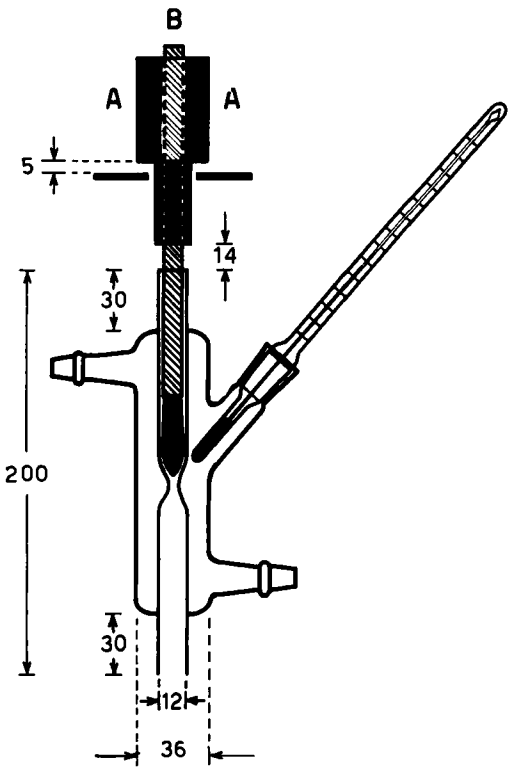

Fig. 1.—Apparatus described in the text for the determination of the softening and liquefaction temperatures. *A*, lead cylinder weighing 460 Gm.; *B*, glass rod, over which a rubber tube is fitted, supporting the lead cylinder. The glass rod and rubber tube have a weight of 40 Gm. The glass rod rests on the suppository. When the suppository collapses the glass rod sinks. After sinking 5 mm. the lead cylinder is supported by a rest and on the suppository there remains only the weight of the glass rod. All measures are in mm. The internal diameter of the glass tube may differ according to the diameter of the suppository. For suppositories of 9.5-mm. diameter, as those used in our experiments, an internal diameter of the tube of 12 mm. is satisfactory.

An alternative method consists in putting on the glass rod, and therefore on the suppository, increasing weights, *e.g.*, lead disks of 100 Gm. In this way the collapsing weight (CW_t) of a suppository may be determined at a given temperature.

When the suppository has collapsed so that it no longer supports the lead cylinder or disks, it bears the weight of the glass rod (30–40 Gm.) only. As the temperature of the water jacket rises, the suppository liquefies, and the glass rod can then sink for another 9 mm. until the rubber rests on the glass tube. We define the temperature at which this happens as the liquefaction temperature (*LT*).

The glass rod is very useful because, as pointed out by Reznek (6) and by Krowczynski (4), an objective measurement of the "melting" or rather of the liquefaction, can be performed only when the suppository is subjected to a light mechanical stress. In our opinion the term "liquefaction temperature" is more proper than that of "melting temperature," since very often suppositories contain drugs which do not melt together with the base and the base, too, may be a mixture of substances with different melting points.

When the suppository has liquefied it flows through the 3-mm. constriction of the glass tube of the apparatus. This usually happens at the liquefaction temperature as defined above. Only for very viscous mixtures is the flow temperature (*FT*) 0.5–1.0° higher than the *LT*.

As described in the result section, we found it convenient to raise the temperature in the apparatus at the rate of 0.1°/min. A simple method of doing this automatically is to drive the thermoregulation knob of a Tecam-Tempunit thermostat water pump with a synchronous motor at the rate of 0.1 angular degrees per minute. Furthermore the sinking of the lead weight or of the glass rod can switch off the current to the motor by means of a microswitch and thus the temperature remains constant when the *ST* or the *LT* is reached.

In order to verify the results obtained, the melting temperature of the bases was determined also by the capillary method for class II substances described in U.S.P. XVI, p. 926 (capillary melting temperature—*CMT*). For water-soluble bases, instead of a water bath, a liquid petrolatum bath was used. In both cases the temperature was raised at the rate of 0.5°/-min.

The melting temperature was controlled by putting the suppositories in a small metallic basket with 5-mm. meshes and keeping these suppositories in an incubator in which the temperature was raised 0.5° every 4 hours. We defined the incubator liquefaction temperature (*ILT*) as the temperature at which at least $^2/_3$ of the suppository had passed through the meshes of the basket.

RESULTS

A very important factor for correct use of the apparatus is the rate at which the temperature is raised in the water jacket. If this rate is too high, the temperature of the suppository remains below that of the water jacket and the temperature read on the thermometer at the moment of softening or of liquefying of the suppository does not correspond to the temperature within the suppository. It is therefore important to define the time necessary to reach thermal equilibrium between the suppository and the water jacket of the apparatus. For this determination we adopted the procedure previously described (1), *i.e.*, we inserted a thermocouple in the middle of a suppository of the shape represented in Fig. 2. The suppository so prepared was then kept for several hours at a temperature of 18 ± 0.2° and then put in the apparatus with the circulating water at 2–3° below the liquefaction temperature of the suppository.

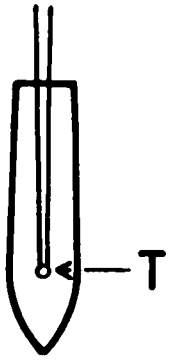

Fig. 2.—Shape and actual size of the suppositories used in our experiments. The position of the thermocouple (T) in the suppository is also shown.

The temperatures measured in the suppository were subjected to a quantitative treatment as previously described. The thermal equilibrium curves obtained on suppositories made with different bases (Fig. 3) show that a 90% thermal equilibrium is reached in 8.5 min. by Carbowax 6000 suppositories, in 12.0 min. by Imhausen H suppositories, in 15.5 min. by theobroma oil suppositories, in 21.5 min. by Neosuppostal suppositories, and in 33.5 min. by Imhausen E suppositories.

On the basis of these data, several procedures of raising the temperature in the apparatus were tried and the following was found convenient. The suppository was put in the apparatus kept at 28.0 ± 0.1° and left at this temperature for 1 hour. Then the temperature was raised at the rate of 0.1°/min. until the ST and the LT were reached. Table I shows the temperature read on the thermometer and the differences between this temperature and the one measured in the middle of the suppository. This difference is not more than 2.0° and, at liquefaction temperature, it becomes very small, probably because heat transmission is improved by the melted part of the suppository which fills the space between the glass tube and the suppository.

Table II gives the capillary melting temperature (CMT), the incubator liquefaction temperature (ILT), the liquefaction temperature (LT), the softening temperature (ST), and the collapsing weight at 25° (CW_{25}) observed in 44 bases or suppositories made with these bases and tested 1–6 months after their preparation.

Table III gives the CMT, the LT, and the ST determined during a 4-month observation on some suppository bases. The same data determined on Carbowax 4000 and on Tween 61 did not show significant changes.

DISCUSSION

Table II shows that there is usually a good agreement between the ILT and the LT, whereas the CMT may be up to 6° lower than the ILT. Since the actual storage conditions of the suppositories are certainly better reproduced by the incubator than by the capillary, the CMT must be considered critically, especially when it is determined on fatty bases and found close to 37°. Indeed, fatty base suppositories liquefy in the rectum only when their liquefaction temperature is below 37° (1). An error of 2–6° in the estimation of the melting temperature means that in some cases a suppository base, which really liquefies above 37° and therefore does not liquefy in the rectum may be accepted as

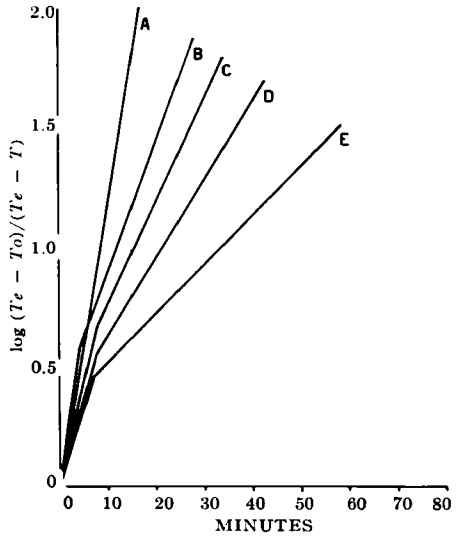

Fig. 3.—Thermal equilibrium curves determined on suppositories obtained with different bases. *Ordinate:* log $(Te - To)/(Te - T)$ (Te is the temperature measured in the suppository at equilibrium, To is the temperature measured at time zero, *i.e.*, at the beginning of the experiment, T is the temperature of the suppository measured at the time corresponding to the abscissa). *Abscissa:* time in minutes. The inclination of the curves expresses the thermal equilibrium rate constant k of the equation: $dT/dt = k(Te - T)$ where t is the time in minutes. A 90% thermal equilibrium is reached in correspondence with $\log (Te - To)/(Te - T) = 1$. A is the curve obtained with a Carbowax 6000 suppository ($k = 0.118$); B, an Imhausen H suppository, ($k = 0.055$); C, a theobroma oil suppository ($k = 0.044$); D, a Neosuppostal suppository ($k = 0.033$); E, an Imhausen E suppository ($k = 0.021$),

TABLE I.—TEMPERATURE IN THE APPARATUS AND DIFFERENCES FROM THE TEMPERATURE IN THE MIDDLE OF THE SUPPOSITORY. TEMPERATURE IN THE APPARATUS WAS INCREASED AT THE RATE OF 0.1°/MIN.

Apparatus	Carbowax 6000	Imhausen H	Theobroma Oil	Imhausen E
28	0	0	0	0
29	−0.1	−0.3	−0.5	−0.1
30	−0.1	−0.7	−0.7	−0.2
31	−0.1	−1.0	−1.0	−0.5
32	−0.1	−1.3	−1.4	−0.9
33	−0.1	−1.5	−2.0	−0.9
34	−0.1	−1.5	−1.0	−1.1
35	−0.1	−0.8	−0.3 (*LT*)	−1.2
36	−0.1	−0.4 (*LT*)		−1.5
37	−0.1			−1.2
38	−0.2			−1.0
39	−0.2			−0.5 (*LT*)
40	−0.2			
45	−0.4			
50	−0.4			
55	−0.5			
60	−0.4			
61	−0.3			
62	−0.2			
63	−0.1 (*LT*)			

satisfactory on the basis of a *CMT* found below 37°. The capillary method has the further disadvantage that some formulations for suppositories, like those containing a large amount of insoluble drugs (*e.g.*, 0.5 Gm. aminophylline in theobroma oil suppositories), are difficult to test since the mixture can not be homogeneously introduced into the capillary. Finally, by this method, it is not possible to determine the melting temperature directly on suppositories, as is necessary, for example, after stability tests, *etc.* For the liquefaction temperature determination, the incubator method and the apparatus described in this paper seems therefore more suitable.

Before choosing the 500-Gm. weight for the determination of the softening temperature, suppositories made with mixtures of theobroma and almond oils were submitted for examination to pharmacists and physicians. The suppositories considered sufficiently firm for handling, storage, and for introduction into the rectum supported a weight between 500 and 700 Gm. (7–10 Gm./mm.^{2}). Since Büchi and Oesch (12) and Malangeau (3) proposed that suppositories of about the same diameter should not be deformed by a weight of 500 Gm., we finally adopted this weight for the determination of the *ST* on our suppositories.

The determination of the *ST* is particularly important for suppositories which must be handled and stored in warm or hot climates. An estimation of the *ST* through the collapsing weight at a given temperature (CW_t), as proposed by Büchi and Oesch (12), is possible only if the two values are very well correlated. The regression calculated on the data obtained on 27 fatty suppository bases is $ST = (24.1 \pm 0.5) + (2.4 \pm 0.33)\, CW_{25}$, from which *ST* can be estimated from the CW_{25} value with an error which may be as much as ±6.7° ($P = 0.05$).

The regression calculated on the data obtained on the 17 water-soluble bases is $ST = (25.88 \pm 1.51) + (5.53 \pm 0.90)\, CW_{25}$, from which *ST* can be estimated with an error which may be as much as ±13.3° ($P = 0.05$). In both cases the correlation of *ST* to CW_{25} is evidently so poor that the determination of the *ST* cannot be replaced by that of the CW_t.

Some comment on the physical qualities of the bases investigated is also relevant. Of the fatty bases, theobroma oil may still be considered as a standard, although it was criticized for its rather low melting temperature and for the comparatively low consistency. On the basis of the *LT* and the *ST* reported in Table II, however, it must be concluded that, of the fatty bases, only Estarinum A, BB, and Pi, Imhausen H, OG, and W, Suppocire A, and Vi-Tin 136, present properties which are as satisfactory as, or better than, those of theobroma oil. However, of the bases with an *LT* higher than 37°, many can be considered for correcting the *LT* of some particular formulations with an *LT* below 35°.

The water-soluble bases, with their high *ST* and *LT*, seem ideally suited for hot climates. But it must be emphasized that these bases liquefy only by a process of dehydrating the rectal mucosa, which is antiphysiological, sometimes irritant and painful, and very often so slow that the drugs vehicled are liberated after only some considerable time (1).

A disadvantage of theobroma oil is its unstable forms, with low melting temperature, which is particularly evident after a heating above 37–40° (13, 14). A consequence of this is that the suppositories remain liquid after pouring in the molds unless these are kept at fairly low temperature. Another consequence is that for the experimenter, it is impossible to know the final physical characteristics of the suppositories for some time after preparation.

In order to investigate the importance of the unstable forms from the analytical point of view, we performed the experiments summarized in Table III. The results show that not only theobroma oil, but also other fatty bases as Dehydag III, Estarinum A and BB, Imhausen H and W, and Suppocire A, reach a stable form not after a few days, as usually stated in the literature, but after 10–30 days. Theobroma oil heated beyond the transition temperature, however, does not reach a stable form even after 4 months. The changes in physical properties are particularly evident in the light of the *CMT* and

TABLE II.—PHYSICAL CHARACTERISTICS OF SOME SUPPOSITORY BASES

Base	CMT,[a] °C.	ILT,[a] °C.	LT,[a] °C.	ST,[a] °C.	CW_{25},[a] Kg.	Supplier
Fatty Bases						
Theobroma Oil	31.6	35	34	29	3.4	.....
Caol TR	39.4	..	39[b]	30	1.6	Sarn, Novara, Italy
Caol TR 38/40	41.7	..	44[b]	34	3.1	" " "
CBSA "N"	36.6	..	37	<18[b]	0.15	Calvè, Delft, Holland
Dehydag III	33.7	40	39[b]	37	>5.0	Deutsche Hydrier-Werke
Dehydag IV	33.7	39	38[b]	36	5.0	" " "
Estarinum A	34.7	37	37	32	4.5	Edelfettwerke, Hamburg
Estarinum B	36.6	39	38[b]	34	5.0	" "
Estarinum BB	34.7	37	36	31	4.2	" "
Estarinum C	38.6	..	40[b]	36	>5.0	" "
Estarinum D	43.3	..	45[b]	41	>5.0	" "
Estarinum Pi	35.0	37	37	34	5.0	" "
Imhausen E	38.2	..	39[b]	33	4.7	Chemische Werke Witten
Imhausen ET	34.7	38	39[b]	33	4.5	" " "
Imhausen H	34.4	36	36	32	4.7	" " "
Imhausen OG	33.5	36	37	29	2.3	" " "
Imhausen W	34.6	37	37	31	4.0	" " "
Imhausen W/N	38.6	..	39[b]	33	3.4	" " "
Lipomassa C.F.M.	38.7	..	39[b]	25[b]	0.5	C.F.M., Milano, Italy
Neo Suppostal	36.5	41	41[b]	28[b]	0.9	Mediframa, Milano, Italy
Suppostal ES	42.2	..	45[b]	32	1.1	" " "
Suppocire A	34.8	36	37	30	2.5	Gattefossè, Lyon
Suppocire B	36.4	38	38[b]	30	3.4	" "
Suppocire C	38.0	..	39[b]	33	4.0	" "
Suppolanol	33.5	..	35	25[b]	0.5	Esperis, Milano, Italy
Vi-Tin 136	34.7	37	37	35	3.9	Treves, Torino, Italy
Vi-Tin 138	36.6	41	39[b]	36	4.3	" " "
Water-soluble Bases						
Carbowax 1540	45.7	..	46	37	2.0	Union Carbide, New York
Carbowax 4000	57.1	..	59	55	>5.0	" " " "
Carbowax 6000	61.6	..	63	56	>5.0	" " " "
Carbowax 6000 + 20% H_2O	58.0	..	61	51	2.0	" " " "
Glycerinated gelatin U.S.P.	48.3	..	50	25[b]	0.5	.....
Idromassa C.F.M.	58.0	..	60	56	4.3	C.F.M., Milano, Italy
Idropostal	58.2	..	60	56	>5.0	Medifarma, Milano, Italy
Idropostal 90% + liquid Idropostal 10%	57.2	..	58	52	>5.0	" " "
Idropostal G 60% + Glicerol 40%	47.0	..	52	37	1.5	" " "
Idropostal M 90% + H_2O 10%	56.2	..	57	49	3.0	" " "
Idrorectonal H	49.6	..	54	41	2.5	Frat. Giacomini, Milano, Italy
Idrorectonal HL	42.0	..	41	30	1.9	" " " "
Idrorectonal W	51.8	..	55	42	4.3	" " " "
Massa Neutralis C.F.M.	39.0	..	39	29	1.3	C.F.M., Milano, Italy
Myrj 52	46.3	..	47	42	>5.0	Atlas Powder
Neutril	57.9	..	59	54	4.9	I.C.V., Como, Italy
Tween 61	40.1	..	39	25	0.5	Atlas Powder

[a] *CMT* = capillary melting temperature; *ILT* = incubator liquefaction temperature; *LT* = liquefaction temperature; ST = softening temperature; CW_{25} = collapsing weight at 25°. [b] Unsatisfactory characteristic.

the *ST* values, though the *LT* temperature reaches a stable value earlier.

The water-soluble bases investigated (Carbowax 4000 and Tween 61) do not show unstable forms.

CONCLUSIONS

As already pointed out by Caldwell (13) the definitions of the suppositories reported in the pharmacopeias, in which it is stated that they should soften, melt, or dissolve at body temperature, are of little assistance unless the method of determining these characteristics is given. In point of fact, different methods give quite different results and sometimes suppositories may be accepted as satisfactory on the basis of the capillary melting temperature for example, even when they do not melt, soften, or dissolve in the rectal environment. Besides this, there should be an accepted method for finding out whether a suppository is sufficiently firm to be introduced in the rectum, handled and stored at ordinary room temperatures or in warm climates.

The method proposed in this paper supplies this information and, for suppositories with fatty bases, shows also whether the liquefaction temperature is sufficiently low to permit liberation of the contained drugs after introduction in the rectum. As a matter of fact all the fatty

TABLE III.—PHYSICAL PROPERTIES OF SOME SUPPOSITORY BASES DURING 120-DAY OBSERVATION

Days		0.1	1	10	20	30	60	120
Theobroma Oil,	CMT	32.2	31.6	33.6	34.3	34.9	35.0	34.6
(heated at 35°C.)	LT		34	34	34	34	35	35
	ST		30	30	30	30	32	32
Theobroma Oil,	CMT	25.1	27.5	27.7	28.0	32.8	32.4	32.0
(heated at 55°C.)	LT		29	29	29	30	34	34
	ST		24	24	24	24	24	30
Dehydag III	CMT	33.3	33.7	35.0	35.0	35.5	36.6	36.9
	LT		36	37	37	38	39	39
	ST		35	35	35	36	37	37
Estarinum A	CMT	32.7	34.7	36.7	36.8	37.5	37.0	36.6
	LT		37	38	38	38	38	37
	ST		31	32	32	31	30	31
Estarinum BB	CMT	32.8	34.7	35.2	35.9	36.7	37.1	37.0
	LT		36	36	36	37	37	37
	ST		32	32	31	30	31	32
Imhausen H	CMT	34.4	34.4	34.6	34.7	34.4	36.7	37.0
	LT		35	36	37	37	37	37
	ST		32	32	32	31	29	29
Imhausen W	CMT	34.2	34.8	35.9	36.0	36.4	36.8	36.5
	LT		36	36	36	37	37	37
	ST		31	31	29	28	28	28
Suppocire A	CMT	34.4	34.8	35.9	36.0	36.5	36.8	36.5
	LT		36	36	36	37	37	37
	ST		31	31	29	28	28	28

bases of Table II with a liquefaction temperature below 37° liquefy in rectal conditions as shown by the results reported by Setnikar and Fantelli (1). But this is not necessarily true for fatty bases with a capillary melting temperature below 37°.

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Proposed Method of Assay for Diaphene

By SOBHI A. SOLIMAN and LOYD E. HARRIS

A rapid and convenient differential spectrophotometric assay is proposed for the assay of Diaphene in liquid soaps and hand creams.

DIAPHENE[1] is a mixture of 5,4′-dibromosalicylanilide and 3,5,4′-tribromosalicylanilide, and is used in toilet detergent formulations. It is reported to possess an unusually long-lasting germicidal effect.

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[1] Trademark of Norton Co., Worcester, Mass. Contributed and marketed by Stecker Chemicals, Inc., Ridgewood, N. J.

Methods described in the literature for the determination of phenolic antiseptics in soaps employ colorimetric or spectrophotometric techniques. The method of Gottlieb and Marsh (1), depending on color formation by the reaction of a phenol with 4-aminoantipyrine in the presence of potassium ferricyanide as an oxidizing agent, was reported to give unreliable results since the color fades rapidly. Ettinger, *et al.* (2), in an attempt to stabilize the color, concentrated the reaction products by extraction with chloroform. This modification, however, does not stabilize the color produced to any degree of accuracy.

The colorimetric method of Johnson and Savidge (3) depends upon the measurement of the

TABLE III.—PHYSICAL PROPERTIES OF SOME SUPPOSITORY BASES DURING 120-DAY OBSERVATION

Days		0.1	1	10	20	30	60	120
Theobroma Oil,	CMT	32.2	31.6	33.6	34.3	34.9	35.0	34.6
(heated at 35°C.)	LT		34	34	34	34	35	35
	ST		30	30	30	30	32	32
Theobroma Oil,	CMT	25.1	27.5	27.7	28.0	32.8	32.4	32.0
(heated at 55°C.)	LT		29	29	29	30	34	34
	ST		24	24	24	24	24	30
Dehydag III	CMT	33.3	33.7	35.0	35.0	35.5	36.6	36.9
	LT		36	37	37	38	39	39
	ST		35	35	35	36	37	37
Estarinum A	CMT	32.7	34.7	36.7	36.8	37.5	37.0	36.6
	LT		37	38	38	38	38	37
	ST		31	32	32	31	30	31
Estarinum BB	CMT	32.8	34.7	35.2	35.9	36.7	37.1	37.0
	LT		36	36	36	37	37	37
	ST		32	32	31	30	31	32
Imhausen H	CMT	34.4	34.4	34.6	34.7	34.4	36.7	37.0
	LT		35	36	37	37	37	37
	ST		32	32	32	31	29	29
Imhausen W	CMT	34.2	34.8	35.9	36.0	36.4	36.8	36.5
	LT		36	36	36	37	37	37
	ST		31	31	29	28	28	28
Suppocire A	CMT	34.4	34.8	35.9	36.0	36.5	36.8	36.5
	LT		36	36	36	37	37	37
	ST		31	31	29	28	28	28

bases of Table II with a liquefaction temperature below 37° liquefy in rectal conditions as shown by the results reported by Setnikar and Fantelli (1). But this is not necessarily true for fatty bases with a capillary melting temperature below 37°.

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Proposed Method of Assay for Diaphene

By SOBHI A. SOLIMAN and LOYD E. HARRIS

A rapid and convenient differential spectrophotometric assay is proposed for the assay of Diaphene in liquid soaps and hand creams.

DIAPHENE[1] is a mixture of 5,4′-dibromosalicylanilide and 3,5,4′-tribromosalicylanilide, and is used in toilet detergent formulations. It is reported to possess an unusually long-lasting germicidal effect.

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Received Honorable Mention, Lunsford-Richardson Pharmacy Award (Eastern Division), 1962.

[1] Trademark of Norton Co., Worcester, Mass. Contributed and marketed by Stecker Chemicals, Inc., Ridgewood, N. J.

Methods described in the literature for the determination of phenolic antiseptics in soaps employ colorimetric or spectrophotometric techniques. The method of Gottlieb and Marsh (1), depending on color formation by the reaction of a phenol with 4-aminoantipyrine in the presence of potassium ferricyanide as an oxidizing agent, was reported to give unreliable results since the color fades rapidly. Ettinger, *et al.* (2), in an attempt to stabilize the color, concentrated the reaction products by extraction with chloroform. This modification, however, does not stabilize the color produced to any degree of accuracy.

The colorimetric method of Johnson and Savidge (3) depends upon the measurement of the

color produced by the coupling of the phenol with 4-aminophenazone in the presence of an alkaline oxidizing agent. The method was reported to give unsatisfactory results with hexachlorophene and dichlorophene since the darkening characteristic of these substances in alkaline medium considerably changes the orange color.

The method of Larson (4), depending on the color determination of the phenol-ferric chloride salt, involves precipitation of the soap with barium bromide. The method was discussed by Childs and Parks (5) who found that the fleeting color produced in this method made it difficult to obtain reproducible results.

The rapid spectrophotometric method of Lord, *et al.* (6), is applied directly to the soap sample by dissolving it in 0.1 *N* potassium hydroxide solution and measuring its absorbance. Measurements were taken at three different wavelengths in the region of the peak of absorption. A correction for irrelevant absorption due to the soap base is made by application of the Morton-Stubbs three-point geometrical correction.

The spectrophotometric procedure of Clements and Newburger (7) is designed for the determination of hexachlorophene in all types of cosmetic formulations and involves several extractions which would appear to be unnecessary for the determination of its content in a liquid soap.

A differential rapid spectrophotometric method was developed by Childs and Parks (5) for the determination of hexachlorophene in the presence of liquid soap. The advantages of this method lie in the fact that the extraction of the phenolic antiseptic from the soap is not necessary; also, it does not depend upon color determination. The method depends on a substance of equal concentration in both the solute and solvent cells of the spectrophotometer when an absorption is obtained that is essentially the same as a cell blank curve. It appeared that such a technique might eliminate the error caused by absorption of the soap base by allowing the instrument to cancel out the irrelevant absorption.

Alkali increases the intensity of absorption of phenols by converting them from the molecular to the ion or salt form, and shifts their absorption peaks to longer wavelengths. Childs and Parks (5) showed that by determining the absorption of hexachlorophene in alkaline media in the solute cell against its absorption in the acid media in the solvent cell, an absorption curve could be obtained which is unique for the phenol and without interference from the soap base. This technique of determining the absorbance of the phenolic compound at an alkaline pH in the solute cell *versus* its same concentration at an acid pH in the solvent cell is known as the differential method.

The differential spectrophotometric procedure has been modified by Matuszak, *et al.* (8), in order to be applicable for the determination of bithionol in liquid soap.

Van Der Pol (9) modified the differential method of Childs and Parks (5) so that it may be used for a wide variety of preparations.

The ion exchange resin method of Suffis and Dean (10) for assaying chlorinated phenolic compounds in soaps depends on separating the antiseptic from the soap and then assaying spectrophotometrically. The separation is based upon the fact that chlorinated phenolics are stronger acids than nonchlorinated phenolics and carboxylic acids. Therefore, they may be separated by their differing adsorptive properties on a strongly basic anionic exchange resin. An empirical calibration must be made using soap preparations of known chlorophenolic content. Apparently resin hold-up prevents a quantitative elution, but a partial and reproducible recovery of chlorophenol is obtained.

The ion exchange resin method was later modified (11) to be applicable for the determination of Diaphene in soap.

EXPERIMENTAL

Apparatus and Reagents.—Beckman DU quartz spectrophotometer with matched 1.0-cm. silica cells; methanol, absolute, reagent grade; 90% methanol, prepared by mixing 100 ml. of distilled water with sufficient anhydrous methanol to make 1000 ml.; hydrochloric acid, approximately 0.1 *N* in distilled water; sodium hydroxide, approximately 0.1 *N* in distilled water.

Development of a Standard Diaphene Spectrophotometric Graph.—Differential absorption spectra of Diaphene (3 mg./100 ml.) in 90% methanol were prepared by measuring the differential absorbance (pH 3 *vs.* pH 8) at various wavelengths. The absorption peak was at 280 mμ. Ninety per cent methanolic solutions of Diaphene (not more than 5 mg./100 ml.) followed Beer's law at this wavelength. The differential absorption spectrum of Diaphene in the presence of soap exhibits a flat peak at 280, 281, and 282 mμ.

Diaphene[2] was assayed at 280 mμ, using the standard absorption graph, in the presence of soap.

A 0.3 *N* acetic acid solution in 90% methanol, was prepared by dissolving 18 Gm. glacial acetic acid in 90% methanol to produce 1000 ml. The solution was then adjusted to pH 3 with 0.1 *N* hydrochloric acid using the Beckman Zeromatic pH meter.

Ninty per cent methanol was adjusted to pH 8 with 0.1 *N* sodium hydroxide using the Beckman Zeromatic pH meter.

Proposed Spectrophotometric Assay for Diaphene.—The differential absorbance index of

[2] ASC-4 brand, marketed by Stecker Chemicals, Inc.

Diaphene in the presence of soap was determined in absorbance units per mg. per ml. as follows: dissolve 5 ml. of the soap base in 90% methanol to make exactly 100 ml.; dissolve a 0.1000-Gm. sample of Diaphene in absolute methanol to make exactly 100 ml.; and dilute a 50-ml. aliquot of this solution to exactly 100 ml. with 90% methanol (solution *A*).

Place 5 ml. of the soap base solution in each of four 100-ml. beakers. To each of the four beakers add 2.0, 3.0, 4.0, and 5.0 ml. of solution *A*. Then to each of the beakers add 20 ml. of 90% methanol and sufficient 0.1 *N* hydrochloric acid to bring the solution to pH 3 on the Beckman Zeromatic pH meter. Wash the electrodes with pH 3 acetic acid–methanol solution. Transfer the beaker contents quantitatively to a dry 50-ml. G. S. volumetric flask, adjust to volume with the acetic acid–methanol solution, and mix thoroughly. This gives a series of solutions containing 2.0, 3.0, 4.0, and 5.0 mg./100 ml. of Diaphene, respectively, at pH 3.

Prepare a second series of solutions of the same concentration as above at pH 8 as follows: Place 5 ml. of the soap base solution in each of four 100-ml. beakers, to each of which add 2.0, 3.0, 4.0, and 5.0 ml. of solution *A*, then 20 ml. of 90% methanol, adjust to pH 8 on a Beckman Zeromatic pH meter using 0.1 *N* sodium hydroxide or 0.1 *N* hydrochloric acid, in distilled water, as required. Wash the electrodes with 90% methanol at pH 8. Transfer the beaker contents quantitatively to a dry 50-ml. G.-S. volumetric flask, adjust to volume with 90% methanol at pH 8 and mix thoroughly. This gives a series of solutions containing 2.0, 3.0, 4.0, and 5.0 mg./100 ml. of Diaphene, respectively, at pH 8.

Arrange the two series of solutions in pairs of the same concentration. Place the pH 3 member of each pair in the solvent cell of the spectrophotometer and the pH 8 member in the solute cell.

Divide the observed absorbance by the number of mg. of Diaphene per ml. of solution to get the absorbance index, I.

The average absorbance index at 280 mμ and slit width of 1.62 mm., as determined by the differential method, should have a value of 10 absorbance units per mg. per ml. in the presence of soap.

TABLE I.—ABSORBANCE INDEX OF DIAPHENE IN 90% METHANOL CONTAINING SOAP SHAMPOO BASE pH 3 *vs.* pH 8[a]

Diaphene, mg. per 100 ml.	Absorbance		Absorbance Index	Av. Absorbance Index
2	0.204	0.204	10.20	
3	0.300	0.300	10.00	
4	0.398	0.400	9.97	
5	0.490	0.495	9.86	10.00

[a] Wavelength, 280 mμ; slit width, 1.62 mm.

Assay Procedure for Diaphene in Liquid Soap.—Dissolve 5 ml. of the soap sample to be assayed in 90% methanol to make exactly 100 ml. (solution *A*). The amount taken could be decreased or increased, but the final dilution should not contain more than 5 mg. Diaphene/100 ml.

Place 5 ml. of solution *A* in a 100-ml. beaker, add 20-ml. of 90% methanol and sufficient 0.1 *N* hydrochloric acid to bring the solution to pH 3 using the Beckman Zeromatic pH meter. Wash the electrodes with pH 3 acetic acid–methanol solution. Transfer the contents of the beaker quantitatively to a dry 50-ml. G.-S. volumetric flask, adjust to volume with acetic acid–methanol solution and mix thoroughly.

Place another 5 ml. of solution *A* in a 100-ml. beaker, add 20 ml. of 90% methanol, adjust to pH 8 on a Beckman Zeromatic pH meter. Wash the electrodes with 90% methanol at pH 8. Transfer the beaker contents quantitatively to a dry 50-ml. G.-S. volumetric flask, adjust to volume with 90% methanol at pH 8 and mix thoroughly.

Measure the absorbance at 280 mμ and slit width 1.62 mm. by placing the pH 3 solution in the solvent cell of the Beckman DU spectrophotometer, and the pH 8 solution in the solute cell.

The per cent of Diaphene was calculated using the following formula:

$$\text{per cent of Diaphene} = \frac{A \times 50 \times 100}{I \times V \times W \times 100}$$

where A = differential absorbance measured; I = differential absorbance index (10); V = volume of solution A in 50 ml. of the assayed solution; W = volume of the sample in ml. × 1000 or weight in mg.

Soap samples to which known amounts of Diaphene had been added were assayed by this method.

Results are given in Table II. The method was also used for the assay of Diaphene in hand cream[3] with results in the same Table.

TABLE II.—DIAPHENE ASSAY IN SOAPS AND CREAMS

	% Diaphene Found
Soap shampoo base plus 0.40% w/v added Diaphene[a]	0.408
Soap shampoo base plus 0.60% w/v added Diaphene	0.602
Soap shampoo base plus 0.80% w/v added Diaphene	0.790
Peck's antiseptic soap base plus 0.70% w/v added Diaphene	0.700
G. S. 40% soap base plus 0.50% w/v added Diaphene	0.502
Hand cream labeled to contain 0.05% w/v Diaphene	0.052
Hand cream labeled to contain 0.05% plus 0.20% added Diaphene	0.253

[a] Diaphene added to the 5-ml. aliquot of the final dilution of the sample.

The Effect of Light and Time on Diaphene.—The effect of sunlight, short ultraviolet, and long ultraviolet light on Diaphene has been studied. The absorbance of 2.5 mg./100 ml. of Diaphene in 90% methanol, containing G. S. 40% soap, at pH 8 was found to decrease gradually on exposure to sunlight for 10 minutes. After that period, the absorbance of the same solution did not show an appreciable change when the solution was exposed to sunlight for a total period of three hours. The solution assumed a distinct yellow color during the 10-minute exposure.

When an acidic solution at pH 3 containing the

[3] In Cuticura Laboratories.

same amount of Diaphene and soap was similarly treated, no significant change in absorbance or color took place.

On exposing a similar solution at pH 8 in a 1.00-cm. silica cell to short ultraviolet light (using a short-wave ultraviolet lamp, model SL 2537, with a short-wave ultraviolet filter, Model SL 2537[4]) a considerable decrease in absorbance took place in a period of 40 minutes after which a gradual change occurred through a total period of three hours. When a pH 3 solution was exposed to the short ultraviolet light, no significant change in absorbance took place in the same period of time.

A long-wave ultraviolet lamp, model SL 3660, with a long-wave ultraviolet filter, model SL 3660[4], was used to determine the effect of long ultraviolet light on Diaphene solutions of pH 8 and pH 3. Absorbances were determined in the same manner (using 90% methanol at pH 8 and pH 3, respectively, in the solvent cell). It was found that long ultraviolet light had more or less the same effect as sunlight on both solutions of Diaphene. No apparent color change took place in either solution after exposure to short or long ultraviolet light, as observed in the 1.0-cm. silica cell. It could be recommended that Diaphene containing preparations that are alkaline should be protected from sunlight as well as ultraviolet light.

DISCUSSION

The spectrophotometric method proposed by Childs and Parks (5) for assaying hexachlorophene in the presence of soap when used for Diaphene gave results that were lower than expected. When liquid soap bases were employed in constructing the standard curve, results were found to be more accurate.

The proposed method eliminates the necessity of the establishment of a standard graph. The method also does not necessitate the presence of the same soap sample free from Diaphene. The proposed spectrophotometric assay for Diaphene was used for assaying liquid soaps and hand creams with accuracy.

[4] Mineralight, marketed by Fisher Scientific Co.

SUMMARY

A rapid, convenient, and accurate differential method for the assay of Diaphene in liquid soaps and hand creams has been proposed. The method is based on the differential spectrophotometric assay for hexachlorophene in liquid soaps developed by Childs and Parks (5).

This proposed method does not necessitate the establishment of a standard graph as was necessary in the Childs and Parks procedure. The method also does not require the same soap base free from Diaphene.

The optical density of Diaphene in solutions at pH 8 is reduced by sunlight and ultraviolet light.

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Use of 3-Azabicyclo[3.2.2]nonane in the Mannich Reaction II. Secondary γ-Amino Alcohols

By C. DEWITT BLANTON, Jr., and W. LEWIS NOBLES

The syntheses of a group of secondary γ-amino alcohols by reduction of the corresponding Mannich base with sodium borohydride are described. These alcohols are to be screened for possible pharmacodynamic activity.

IN THE FIRST paper of this series (1), a number of substituted β-amino ketones were synthesized for pharmacological evaluation employing the Mannich reaction and 3-azabicyclo[3.2.2]-nonane as the amine moiety. Previously, Denton (2) and his associates had similarly prepared certain amino ketones and converted them to the corresponding secondary and tertiary alcohols. In many cases, the conversion to the alcohol had a significant effect on the physiological activity. Therefore, it was considered that the transformation of the ketones previously reported by us (1), might possibly have a similar effect. In

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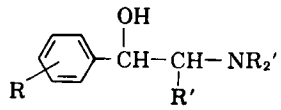
addition, the γ-amino alcohols are generally more stable than the corresponding ketones.

An examination of the ring system utilized in these studies will indicate a relationship to the complex heterocyclic systems which occur in certain natural products, such as cocaine and atropine. Thus, in every instance, caged structures are present in the molecule. Furthermore, it may be noted that the ring systems in cocaine may be regarded as a condensed piperidine-pyrollidine nucleus whereas the present amine component, 3-azabicyclo[3.2.2]nonane may be considered to be a cyclohexane ring condensed with a hexamethyleneimine moiety. Similarly, this system may be compared with many derivatives of the solanaceous alkaloids which also possess the condensed piperidine-pyrollidine nucleus.

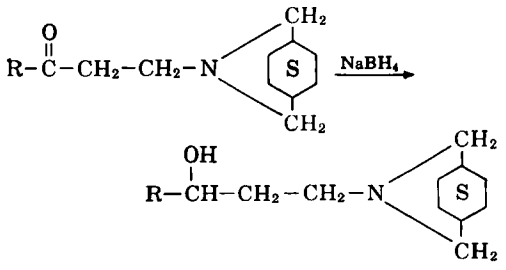
Lutz and co-workers (3) have reported the preparation and screening of 184 amino alcohols against avian malaria. The general structure of the secondary alcohols is represented as



These compounds included examples of 50 variations in the benzene nucleus and over 60 variations in the N,N-dialkyl groups on the nitrogen. Although most of these amino alcohols proved to be inactive, special mention should be made of the α-dichlorophenyl-β-dioctylaminoethanols which proved to be equal to or better than quinine in tests against avian malaria.

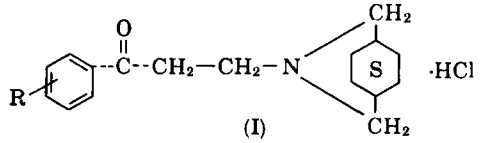
Denton and his associates (4) reported the preparation of a secondary alcohol, 3-(1-piperidyl)-1,2-diphenyl-1-propanol hydrochloride, and noted that it ranked higher in antispasmodic activity than did the seven tertiary alcohols with which it was compared. 3-Dimethylaminophenyl-1-propanol hydrochloride was prepared by Denton and co-workers (5) and was found to rank low in antispasmodic activity when compared with 15 tertiary alcohols.

We have prepared a series of secondary alcohols from the substituted β-amino ketones previously reported (1) according to the reaction

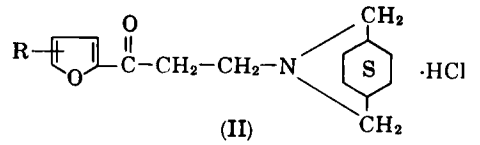


Our results are reported in Tables I and II.

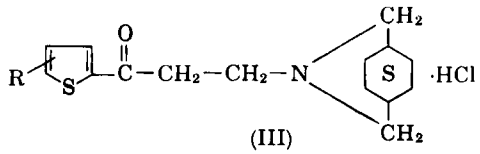
During the course of this work, compounds having the general structure of types I and III were converted to alcohols without difficulty. Under these conditions, treatment of compounds of type II did not give the expected secondary alcohol.



(I)



(II)



(III)

It is possible as Elderfield (6) reports that the furan ring is more easily reduced than the benzene ring to give the tetrahydrofurfuryl alcohol together with smaller amounts of the products of reductive ring opening: pentanediols-1,2, and -1,5, and pentanol-1.

PHARMACOLOGICAL RESULTS

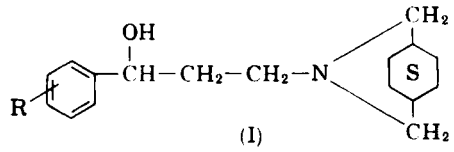
Preliminary screening data (7) indicate a weak central nervous system activity for compound 16 (Table I). This activity was characterized by hypotonia, salivation, and fore-limb clonus. Compounds 1 and 2 of Table I and compound 1 of Table II failed to produce any overt activity when administered orally in doses of 300 mg./Kg. As other results become available, it is anticipated that they will be reported as an integral part of this overall study.

EXPERIMENTAL

The preparation of the γ-amino secondary alcohols was patterned after the method of Chaikin and Brown (8) as employed by Rogers and Nobles (9). These secondary alcohols were all prepared by one of the following two procedures. Basically, as will be evident from an inspection of the actual procedures, these methods differ only in regard to the manner in which the final product is isolated.

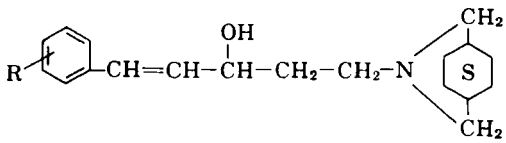
Procedure A.—The γ-amino secondary alcohols were prepared by the general procedure of suspending 0.05 mole of the β-amino ketone hydrochloride in 100 ml. of distilled water and making the solution basic to litmus with 10% sodium hydroxide solution. The free Mannich base was then collected. If this was a solid, it could be filtered off. If the free base was a liquid, it could be collected by extraction with ether. The Mannich base was dissolved in 100 ml. of methanol. This alcoholic solution was placed in a 300-ml. three-neck flask fitted with a dropping

TABLE I.—SECONDARY γ-AMINO ALCOHOLS

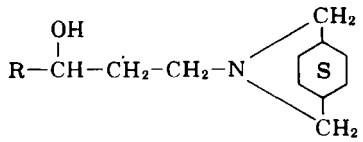


(I)

No.[a]	R	Yield, %	M.p.,[b] °C.	Formula	Analyses[c] Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found
1	Hydrogen	...	241–242	$C_{17}H_{25}NO.HCl$	69.08	68.66	8.87	9.14	4.74	4.96
2	*p*-Nitro	80	113–114	$C_{17}H_{24}N_2O_3$	67.10	67.11	7.89	8.02	9.21	9.39
3	*p*-Methoxy	79	93–94	$C_{18}H_{27}NO_2$	74.74	74.84	9.34	9.47	4.84	5.24
4	*p*-Ethoxy	84	88–89	$C_{19}H_{29}NO_2$	75.25	74.96	9.51	9.55	4.64	4.59
5	*p*-Chloro	93	104–105	$C_{17}H_{24}ClNO$	69.51	69.66	8.18	8.17	4.77	4.79
6	*p*-Methyl	85	98–99	$C_{18}H_{27}NO$	79.07	79.23	9.95	10.22	5.12	5.13
7	*p*-Fluoro	49	49–50	$C_{17}H_{24}FNO$	73.61	73.55	8.72	8.93	5.05	5.25
8	*p*-Phenyl	82	117–120	$C_{23}H_{29}NO$	82.34	81.71	8.71	8.63	4.17	4.34
9	*p*-Bromo	89	108–109	$C_{17}H_{24}BrNO$	60.36	60.88	7.15	7.15	4.14	4.15
10	*m*-Nitro	68	213–214	$C_{17}H_{24}N_2O_3.HCl$	59.90	59.94	7.39	7.56	8.22	8.31
11	*p*-Ethyl	92	85–86	$C_{19}H_{29}NO$	79.39	79.51	10.17	10.52	4.87	5.03
12	*o*-Hydroxy	34	164–165	$C_{17}H_{25}NO_2.HCl$	65.49	65.35	8.35	8.09	4.49	4.65
13	*p*-Hydroxy[d]	76	72–75	$C_{17}H_{25}NO_2$	69.62	69.98	9.22	9.24	4.78	5.08
14	*m*-Bromo	59	233–234	$C_{17}H_{24}BrNO.HCl$	54.47	54.69	6.68	6.77	3.74	3.83
15	*m*-Hydroxy	88	137–138	$C_{17}H_{25}NO_2$	74.18	74.32	9.09	9.84	5.09	5.11



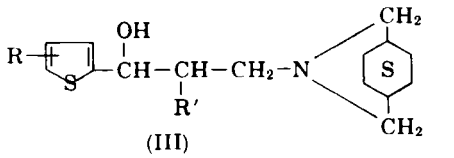
16	Hydrogen	72	78–79	$C_{19}H_{27}NO$	79.95	80.09	9.53	9.33	4.91	4.79
17	*p*-Methoxy	83	84–85	$C_{20}H_{29}NO_2$	76.19	75.97	9.21	9.19	4.44	4.15
18	*p*-Chloro[e]	22	178–180	$C_{19}H_{26}ClNO.HCl$	61.71	61.78	7.77	7.69	3.79	3.57



19	1-Naphthyl	43	213–214	$C_{21}H_{27}NO.HCl$	72.92	73.05	8.16	8.10	4.05	4.25
20	2-Naphthyl	59	217–218	$C_{21}H_{27}NO.HCl$	72.92	73.14	8.15	8.13	4.05	4.03

[a] All secondary γ-amino alcohols were recrystallized from an ethanol-water or ethanol-acetone solution. [b] Melting points are uncorrected. [c] Carbon, hydrogen, and nitrogen analyses are by Smith Kline & French Laboratories, Philadelphia, Pa. [d] Calculated for one mole of water. [e] Calculated for three-fourths mole of water.

TABLE II.—SECONDARY γ-AMINO ALCOHOLS



(III)

No.[a]	R	R′	Yield, %	M.p.,[b] °C.	Formula	Analyses[c] Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found
1	Hydrogen	Hydrogen	42	221–222	$C_{15}H_{23}NOS.HCl$	59.70	59.63	7.96	7.98	4.65	4.44
2	Hydrogen	Methyl	69	232–233	$C_{16}H_{25}NOS.HCl$	60.83	60.53	8.30	8.40	4.43	4.32
3	5-Bromo	Methyl	34	240–242	$C_{16}H_{24}BrNOS.HCl$	48.67	48.98	6.34	6.36	3.55	3.46

[a] All secondary γ-amino alcohols were recrystallized from an ethanol-water or ethanol-acetone solution. [b] Melting points are uncorrected. [c] Carbon, hydrogen, and nitrogen analyses are through the courtesy of Dr. Paul Craig of Smith Kline & French Laboratories, Philadelphia, Pa.

funnel, thermometer, reflux condenser, and magnetic stirrer. To this was added 0.1 mole of sodium borohydride dissolved in 50 ml. of methanol. The addition was conducted at such a rate as to maintain the temperature between 20–40°. After the evolution of hydrogen had subsided somewhat, the methanol was removed under water-pump vacuum. The residue was suspended in 100 ml. of distilled water and extracted with three 100-ml. portions of ether. The ether was then removed under diminished pressure and the solid material recrystallized to analytical purity from an ethanol-water solution.

Procedure B.—This method was utilized only when the secondary alcohol obtained upon removal of the ether was an oil. The liquid alcohols obtained by Procedure A were dissolved in 100 ml. of anhydrous ether. This ether solution was treated with anhydrous hydrogen chloride and the ether decanted from the sticky mass which adhered to the sides of the flask. A few milliliters of acetone was added and shortly a white solid appeared. This solid material was recrystallized to analytical purity from an ethanol-acetone solution.

Compound number 19 from Table I was prepared from the corresponding ketonic Mannich base. This Mannich base was prepared from 1-acetonaphthone in a 31% yield according to procedures previously indicated (1). After recrystallization to analytical purity from an ethanol-acetone solution, a m.p. of 219–221° was observed.

Anal.—Calcd. for $C_{21}H_{25}NO \cdot HCl$: C, 73.34; H, 7.62; N, 4.07. Found: C, 73.53; H, 7.59; N, 4.06.

REFERENCES

(1) Blanton, C. D., and Nobles, W. L., THIS JOURNAL, **51**, 878(1962).
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Kinetics of Rapid Aggregation in Suspensions

Comparison of Experiments with the Smoluchowski Theory

By W. I. HIGUCHI, R. OKADA, G. A. STELTER, and A. P. LEMBERGER

Rates of aggregation of initially monodispersed 1.83-μ polystyrene latex particles in various electrolyte solutions at different electrolyte concentrations have been studied. The experiments involved the determination of the distribution of singlets, doublets, and triplets as a function of time with the Coulter Counter. In order to permit a comparison of the data with theory, theoretical calculations of the "bimolecular" rate constants were carried out for both the diffusion-controlled mechanism and the surface-controlled mechanism. These rate constants were employed to construct theoretical curves which were used to evaluate the data. Maximum rates observed approached the Smoluchowski rate to within about a factor of two. With the purified samples the rates were found to be relatively independent of electrolyte concentration and type. Rates observed with the unpurified samples in salt solutions were substantially lower than those for the purified suspension even at high salt concentrations.

MOST STUDIES (1–4) of aggregation in solid-liquid or liquid-liquid dispersed systems have involved methods based on observation of the sedimentation behavior. While in many instances the desired information may be or may best be obtained by these techniques, it is generally difficult to quantitate aggregation, *per se*, from such experiments.

With the introduction of a novel instrument, the Coulter Counter,[1] it has become possible to conveniently study aggregation without the complicating effects of sedimentation. Recently (5) the reversible aggregation in oil-in-water emulsions was studied with the aid of this instrument. Considerable insight into the problem was gained from this investigation.

In the present communication, results of a study of the kinetics of rapid aggregation of initially monodispersed polystyrene latex suspension particles are reported. The purposes of this work were to evaluate the validity of the existing theories as they apply to rapid aggrega-

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TABLE I.—THE IRREVERSIBLE AGGREGATION INVOLVING UNIFORM-SIZED PRIMARY PARTICLES

$A_1 + A_1 \xrightarrow{k_{11}} A_2$	...	...	...	...
$A_1 + A_2 \xrightarrow{k_{12}} A_3$	$A_2 + A_2 \xrightarrow{k_{22}} A_4$	...	...	...
$A_1 + A_3 \xrightarrow{k_{13}} A_4$	$A_2 + A_3 \xrightarrow{k_{23}} A_5$	$A_3 + A_3 \xrightarrow{k_{33}} A_6$	...	...
$A_1 + A_4 \xrightarrow{k_{14}} A_5$	$A_2 + A_4 \xrightarrow{k_{24}} A_6$	$A_3 + A_4 \xrightarrow{k_{34}} A_7$	$A_4 + A_4 \xrightarrow{k_{44}} A_8$	...
Etc.	Etc.	Etc.	Etc.	Etc.

tion of particles in the micron size range and to lay the groundwork for future studies on the role of additives in influencing the aggregation behavior in dispersed systems.

THEORY

We shall concern ourselves with the problem of the rapid irreversible aggregation of an initially monodispersed suspension. It is assumed that stirring is not present and that the effects of sedimentation are negligible. The reactions which must be considered are given in the scheme in Table I. Here the A_1, A_2, *etc.*, refer to the singlet, doublet, *etc.*, and the k's are the rate constants for the reactions. The differential equations for this set of bimolecular reactions may be written

$$\frac{d(A_1)}{dt} = -(A_1)[k_{11}(A_1) + k_{12}(A_2) + k_{13}(A_3) + k_{14}(A_4) + \ldots]$$

$$\frac{d(A_2)}{dt} = {}^1/_2 k_{11}(A_1)^2 - (A_2)[k_{12}(A_1) + k_{22}(A_2) + k_{23}(A_3) + k_{24}(A_4) + \ldots]$$

$$\frac{d(A_3)}{dt} = k_{12}(A_1)(A_2) - (A_3)[k_{13}(A_3) + k_{23}(A_2) + k_{33}(A_3) + k_{34}(A_4) + \ldots]$$

$$\frac{d(A_4)}{dt} = k_{13}(A_1)(A_3) + k_{22}(A_2)^2 - (A_4)[k_{14}(A_1) + k_{24}(A_2) + k_{34}(A_3) + k_{44}(A_4) + k_{45}(A_5) + \ldots]$$

$$\vdots$$

etc. (Eq. 1)

In these equations (A_1), (A_2), *etc.*, are the concentrations of A_1, A_2, *etc.*, in number of particles per ml. and t is time in seconds. These equations cannot be analytically solved for the general case. However, for the special case in which a steady-state[2] diffusion is assumed and in which all of the rate constants, k's, are equal, the solutions may be easily obtained (6). Since, as we shall see later, the rate constants may not differ too greatly for the aggregate pairs under consideration in our experiments, the special case of all k's being equal is a useful approximation.

For cases in which the rate constants are different, the equations may be solved numerically by the usual methods (8). There are two of these cases which are of particular interest to our problem. One of these is the diffusion-controlled (no particle-particle repulsion) case and the other is the surface-barrier-controlled case. In the diffusion-controlled process, the rate is determined by how rapidly the aggregates can diffuse field-free to each other by Brownian motion. Thus this rate is the theoretical maximum rate.[3] The other case of interest is that in which aggregation occurs much more slowly. This may come about if a stabilizing agent acting at the particle surface is able to reduce the effectiveness of particle-particle sticking during collisions between the particles. In this instance the rate will be determined principally by not how quickly the particles can diffuse to each other, but by how rapidly they can overcome the barrier between them after they have nearly gotten together.

The rate constants, k's, for the surface-controlled process should be proportional to the interfacial contact area between the aggregates. For the diffusion-controlled rate constants this is not the case (6, 7). This difference is the reason for the difference in the aggregate size dependence upon the rate constants between the two processes.

TABLE II.—THEORETICAL RATE CONSTANTS FOR DIFFUSION-CONTROLLED AND SURFACE-CONTROLLED PROCESSES

Rate Constant	D. C.	S. C.
k_{11}	k_{11}	k_{11}
k_{12}	1.03 k_{11}	1.3 k_{11}
k_{13}	1.07 k_{11}	1.5 k_{11}
k_{14}	1.13 k_{11}	1.7 k_{11}
k_{15}	1.16 k_{11}	1.8 k_{11}
k_{22}	1.00 k_{11}	1.5 k_{11}
k_{23}	1.01 k_{11}	1.7 k_{11}
k_{24}	1.03 k_{11}	1.9 k_{11}
k_{33}	1.00 k_{11}	1.8 k_{11}
k_{34}	1.0 k_{11}	2.0 k_{11}
k_{44}	1.0 k_{11}	2.2 k_{11}

The results of the theoretical calculations[4] of the aggregate size dependence upon the rate constants for these two cases are given in Table II. For these calculations of the rate constants in the diffusion controlled case we started with Smoluchowski's relation (6, 7)

$$k_{ij} = 4\pi(D_i + D_j)(R_i + R_j) \quad \text{(Eq. 2)}$$

[2] See Overbeek, Reference 7, for the validity criterion for this assumption. This steady-state is not to be confused with "Steady-state" concentration of aggregates.

[3] Actually if the theory includes the effects of the attractive long-range London dispersion forces, calculations would predict greater (by the order of about 1 to 10%) maximum rates for particles in the micron size range. See Reference 9 for a discussion of these forces.

[4] Unpublished work. Because of the approximate nature of these calculations, only the outline of the method of computation is presented here.

where D_i and D_j are the diffusion coefficients for the i and j aggregates, and R_i and R_j are the respective "collision" radii. The sum, $D_i + D_j$, is the effective diffusion coefficient for the pair, and the sum, $R_i + R_j$, is the interparticle distance (center-to-center) at particle-particle contact. While Eq. 2 is well suited for calculations involving spheres only, it cannot be rigorously applied where nonspheres are involved. In the latter cases approximations become necessary. Thus the singlet-singlet rate constant, k_{11}, was directly obtained by combining Eq. 2 with the Stokes-Einstein relation for a sphere

$$D_1 = \frac{k_o T}{6\pi\eta a} \qquad \text{(Eq. 3)}$$

where k_o is Boltzmann's constant, T is the absolute temperature, η is the viscosity, and a is the radius of the primary particle. Thus

$$k_{11} = \frac{8k_o T}{3\eta} \qquad \text{(Eq. 4)}$$

for the diffusion-controlled case. For calculations involving doublets, the translational diffusion coefficient for the doublet was calculated with the assumption that the doublet is a prolate spheroid with an axial ratio of two with the minor axis equal to the diameter of the singlet sphere. Then the equations of Perrin (10) were used to estimate the D value assuming random orientation of the doublet. The effective collision radius, R, for the doublet was obtained using this spheroid model and finding the radius of the equivalent volume[5] sphere. For triplets and quadruplets and D and R values were estimated in the same manner but the various arrangements of the primary particles were weighted. For aggregates made up of more than four particles it was assumed that the aggregates were spheres with hydrodynamic radii equal to the radii of spheres of equal mass but with a porosity of 0.5. In all of these calculations the effects of rotational diffusion were neglected.

The rate constant calculations for the surface-controlled case involved primarily the estimation of the available areas of contact between the two aggregates involved. We may write

$$k_{ij} = (\text{Total Available Contact Area})(D_i + D_j)\,\alpha \qquad \text{(Eq. 5)}$$

where α is a constant for a given system in which the local curvature of the contacting surfaces are the same.[6] The factor includes the sticking probability determined by the barrier. As before the D's in Eq. 5 are the diffusion coefficient of the aggregates. These were calculated in the same manner as those for the diffusion-controlled process. The total contacting area for the singlet-singlet is simply $16\pi a^2$. Therefore

$$k_{11} = 32\pi a^2 D_1 \alpha \qquad \text{(Eq. 6)}$$

With Eq. 3 this becomes

$$k_{11} = \frac{16 k_o T a \alpha}{3\eta} \qquad \text{(Eq. 7)}$$

for the surface-controlled case. The contact areas for the multiplets were estimated by calculating the surface generated by the center of one aggregate around the other with aggregate-aggregate contact always present. Equivalent volume sphere approximations based on the spheroidal model were used as in the diffusion-controlled case for the calculations involving doublets and triplets. The larger aggregates were assumed as before to be spheres of equal mass with a porosity of 0.5. Again rotational diffusion of the aggregates was ignored.

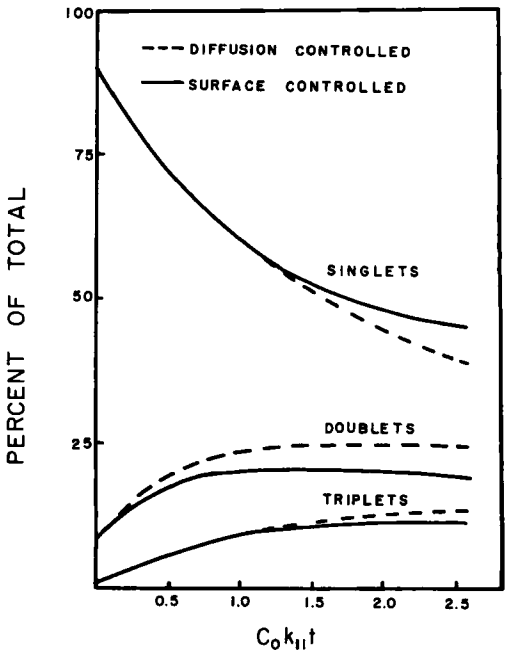

Fig. 1.—Theoretical curves for the per cent of various species as a function of time in a system composed of uniform-size primary particles undergoing irreversible aggregation at zero shear and zero sedimentation.

In Fig. 1 the results of the numerical integration of Eq. 1 employing the k values in Table II are presented. Where necessary the rate constants given in Table II were supplemented with k's involving larger (than four) aggregates calculated by the methods outlined above. For the calculations over the range of $C_o k_{11} t$ given in Fig. 1, only the rate constants involving small aggregates were of major importance. The particular boundary conditions selected for the integrations were at $t = o$

$$\begin{aligned}
\%\ \text{singlets} &= \frac{100\,(A_1)}{(A_T)} = 90 \\
\%\ \text{doublets} &= \frac{100\,(A_2)}{(A_T)} = 8 \qquad \text{(Eq. 8)} \\
\%\ \text{triplets} &= \frac{100\,(A_3)}{(A_T)} = 1 \\
\%\ \text{larger aggregates} &= \frac{100 \sum_4^\infty (A_i)}{(A_T)} = 1
\end{aligned}$$

where the total particle number concentration is

[5] This equivalent volume sphere calculation compares well with that employing rigorous equations obtained from electrostatic theory (11) with the spheroid model.

[6] This is generally expected for aggregates composed of uniform size spherical primary particles.

$$(A_T) = \sum_{i=1}^{\infty} (A_i) \text{ at any } t$$

and

$$C_o = (A_T) \text{ at } t = 0$$

This set of conditions was chosen because it approximately conforms to most of the experiments which will be discussed later.

It can be seen from Fig. 1 that according to theory the dependence of the number distribution of aggregate sizes upon $C_o k_{11} t$ does not differ too greatly for the two processes over the range considered. As expected, the initial portions of the two curves are identical. The singlet curve for the surface-controlled case tends to level off more rapidly than that for the diffusion-controlled case at large times. Also the doublet and triplet curves for the surface-controlled case do not reach as high as those for the diffusion-controlled case.

When data conform to the plots in Fig. 1, it is simply a matter of matching the data curve to the theoretical curves. Then the k_{11} may be determined directly if C_o is known. The experimental k_{11} values may be compared then with Eq. 4 to see whether the system obeys the Smoluchowski theory or, if not, to see how much stabilization (retardation of aggregation) is present.

Because of the near constancy of the k values for the diffusion-controlled case (see Table II), equating all of the k's to k_{11} results in a good approximation for this case at small times. As already mentioned, analytical solutions to Eq. 1 exist (6, 7) for this situation. If at time = 0 there are 100% singlets present, then for later times this approximation gives

$$\% \text{ singlets} = \frac{100}{1 + (1/2) C_o k_{11} t}$$

$$\% \text{ doublets} = \frac{50\, k_{11} C_o t}{[1 + (1/2) C_o k_{11} t]^2} \quad \text{(Eq. 9)}$$

$$\% \text{ triplets} = \frac{100\, [(1/2)\, k_{11} C_o t]^2}{[1 + (1/2)\, k_{11} C_o t]^3}$$

Plots of these equations are essentially identical with those for the diffusion-controlled case given in Fig. 1. The simplicity of Eq. 9 makes them useful when curves of the type given in Fig. 1 are not available.

EXPERIMENTAL

General Considerations.—While it is generally conceded (6, 7) that the Smoluchowski theory explains the data fairly well for the limiting coagulation rates of sols in the 0.01 to 0.1-μ size range, there appears to have been no examination of the applicability of the theory to particles in the micron-size range. Because particle sizes in most pharmaceutical suspensions range mainly in the micron region, mass-wise, a basic understanding of suspension aggregation behavior in this size region is important. To this end, the questions of whether and when the Smoluchowski theory applies are foremost and essential.

The system selected for this study was the suspension of monodispersed polystyrene latex particles[7] of 1.83-μ diameter. For this system the resolution of the Coulter Counter is sufficiently great as to permit the classification of the aggregates into singlets, doublets, triplets, and sometimes even quadruplets. This is illustrated for a typical case of a partially aggregated system in Fig. 2. The differences between the plateau values on the curve give the relative amounts of the different species. Thus it was possible to conveniently follow the time change in population of the various aggregates in the suspension.

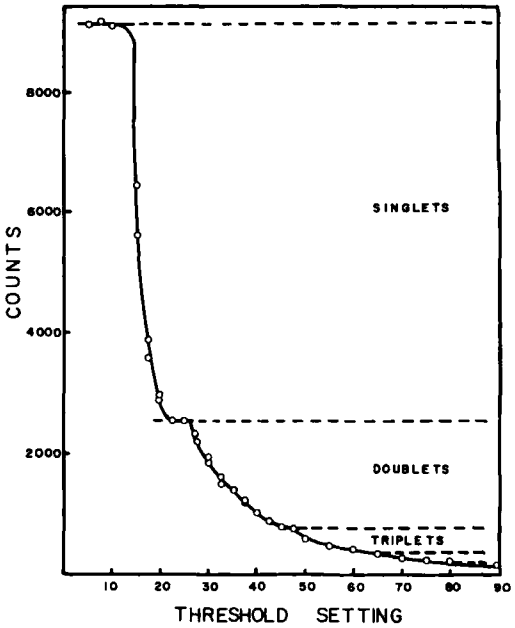

Fig. 2.—Coulter Counter data obtained with a system of partially aggregated suspension composed of 1.83-μ diameter primary particles.

It was decided to carry out the kinetic studies on both a purified suspension as well as on the unpurified sample in the presence of various electrolytes at different electrolyte concentrations. Since it was known (12) that the soluble impurity in the sample as received was a sulfonate type stabilizer, the limiting aggregation rates for the unpurified suspension were expected to also approach the theoretical Smoluchowski rate if the theory was to apply in this instance.

Procedure.—The sample of the latex suspension was diluted to about 1×10^8 particles per ml. A portion of this master suspension was purified by centrifugation in a clinical centrifuge followed by decantation of the liquid and ultrasonic[8] redispersion in the solvent. This was repeated three times in 70% aqueous ethanol and three times in redistilled water. The other portion of the master suspension was used directly without any purification.

For the runs the suspensions were further diluted and mixed with equal volumes of an aqueous electrolyte solution so that the initial total particle concentration, c_o, was always in the neighborhood of 1×10^7 to 7×10^7 particles per ml. These suspen-

[7] Kindly supplied to us by Dr. J. W. Vanderhoff, Dow Chemical Co., Midland, Mich.

[8] Ultrasonic cleaning unit, model DR-125AH, Acoustica Associates, Mineola, N. Y.

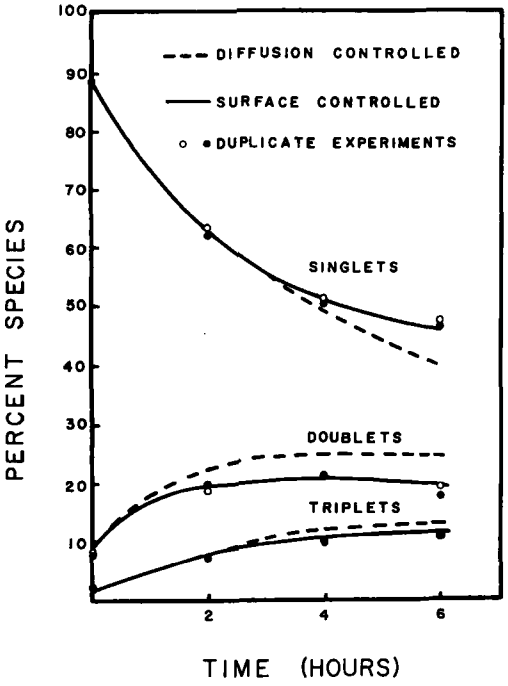

Fig. 3.—Experiment and theory showing aggregation of purified polystyrene suspension particles in 1% $MgSO_4$. $C_o = 1.7 \times 10^7$ particles per ml.

sions were then allowed to stand without agitation[9] in 25-ml. volumetric flasks maintained at 25.0 ± 0.5°. Aliquots were taken periodically, diluted in 0.9% sodium chloride solution, and the distribution of aggregates were determined with the Coulter Counter employing the 50-μ aperture. The dilutions for counting were sufficiently great so that coincidence effects were negligible. Generally about 1 ml. of the suspension was added to 100 to 250 ml. of saline. The manner of pipeting and mixing the aliquot was found not to influence the results.

RESULTS AND DISCUSSION

Time Dependence.—The results of two of the kinetic runs are presented in Figs. 3 and 4. The data are plotted as per cent species *vs.* time to permit comparison with theory. For the particular experiment the theoretical curves were obtained by selecting a suitable single value for k_{11} and replotting the curves in Fig. 1 with the experimental C_o so that the singlet theoretical curve would give the best fit to the singlet data.

For these and for the other electrolyte systems the fit of the data was more often somewhat better for the surface-controlled theoretical case, but in some instances the fit was equally good for both theoretical cases. As we shall see later, the Smoluchowski rate constant for the singlet-singlet reaction as predicted by Eq. 4 was never reached in any of the experiments, although closely approached particularly by the runs with the purified suspension and by the runs in 1.0% hydrochloric acid with the unpurified suspension (Fig. 4). Thus it is reasonable that most of the data did fit the surface-controlled theory or fit somewhere in between the two. However, because in many cases the experimental uncertainty was not much less than the differences between the two theoretical predictions, no conclusions can be made regarding the correct mechanism on this basis alone. Up to this point, however, it can be said that the time-dependent behavior of the aggregate distribution was found to be in good agreement with theory.

Dependence of k_{11} on Electrolyte Concentration.—By means of the method described above for matching theoretical curve to data, values for k_{11} were determined for all of the runs. The results are presented in Figs. 5 and 6 for the purified and the unpurified suspensions, respectively.

It is first worthwhile to note from these results that the k_{11} value as predicted by the diffusion controlled theory of Smoluchowski (see Eq. 4), *viz.* $k_{11} = 1.2 \times 10^{-11}$, has been closely approached in most of these experiments. In particular, with the purified suspension, all salt systems gave maximum k_{11} values within about a factor of two of the theoretical value. Because it is believed that these latex particles are typically hydrophobic, similar maximum k_{11} values can be expected with most unprotected suspensions and emulsions. Thus a useful upper limit for rate of aggregation has been experimentally established.

The salt runs with the unpurified suspension consistently gave lower k_{11} values and showed greater concentration dependence in the range of study than the runs with the purified sample. While it was expected that the concentration dependence would

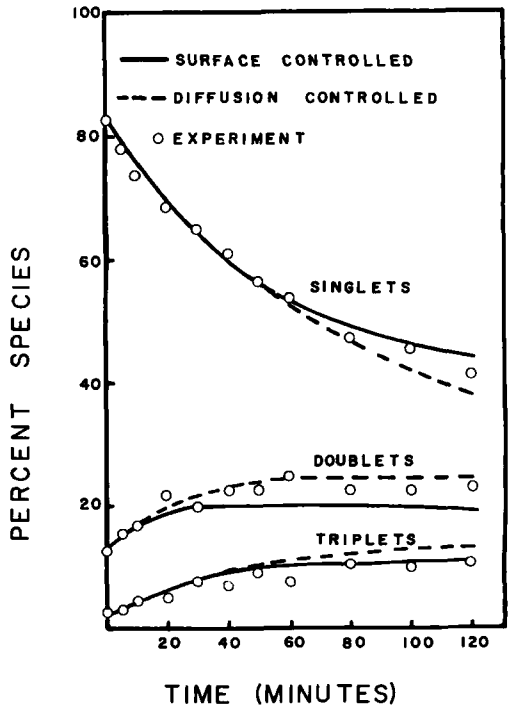

Fig. 4.—Experiment and theory showing aggregation of unpurified polystyrene latex suspension particles in 1% HCl. $C_o = 5.2 \times 10^7$ particles per ml.

[9] Both from Stokes' law consideration and actual experimental observations, sedimentation effects were judged to be negligible during the experiments.

be generally greater for the unpurified suspension because of the stabilizing action of the sulfonate additive at low salt concentration, it was somewhat surprising to find that maximum k_{11} values nearer to the theoretical k_{11} were not obtained in this series of runs. A sulfonate would normally be expected to operate as a charge-conferring agent. At such high salt concentrations (1 to 5%) the electrical double layer would be very thin and therefore should be completely canceled out at all interparticle distances by the attractive long-range London dispersion forces (7) between the primary particles. Thus it was expected that repulsive interactions would be entirely absent. The real situation is perhaps more complex than this simple picture. It may possibly involve aggregation of the sulfonate in a gel-like structure at the particle surface.

The effect of hydrochloric acid on the aggregation rate of the unpurified suspension was most interesting (see Fig. 6). The protective action of the stabilizer appeared to have been nearly completely destroyed in 1.0% hydrochloric acid. At this hydrochloric acid concentration the k_{11} was found to be approximately the same as those limiting values found with the purified suspension. The decrease in rate at higher hydrochloric acid concentrations has not been explained.

The dependences of k_{11} upon both the electrolyte type and the electrolyte concentration were relatively small with the purified suspension. This was not surprising since the few charge-producing ions present in these systems were not expected to

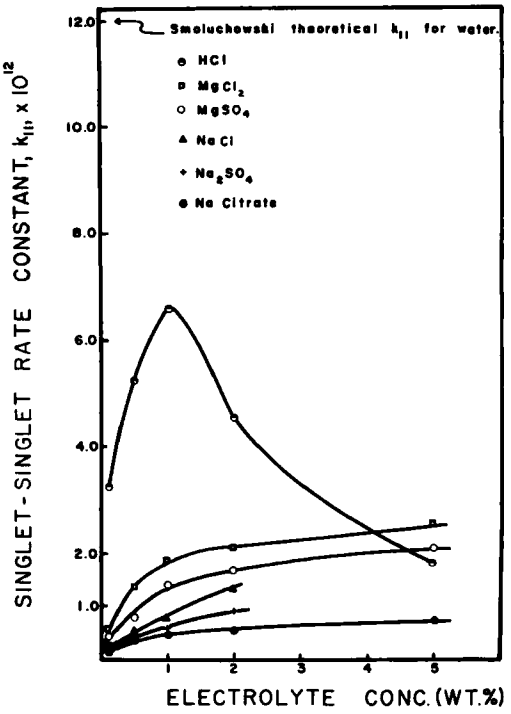

Fig. 6.—Experimental k_{11} values obtained with unpurified polystyrene latex suspension as function of electrolyte type and concentration. Theoretical $k_{11} = 1.2 \times 10^{-11}$.

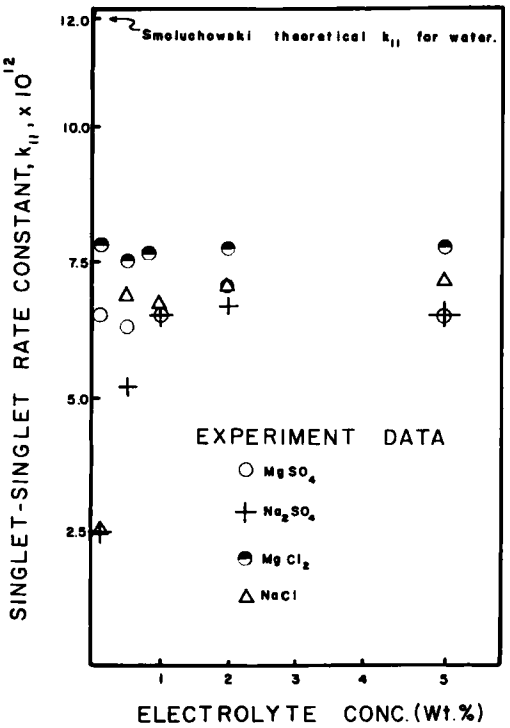

Fig. 5.—Experimental k_{11} values obtained with purified polystyrene suspension as function of electrolyte type and concentration. Note theoretical $k_{11} = 1.2 \times 10^{-11}$.

be very effective. To within about ±10%, the maximum k_{11} values observed in this series were found to be the same in all electrolyte systems studied.

While it may be purely academic, it is interesting to note that these experiments suggest that the correct diffusion-controlled rate is somewhat less than that predicted by the Smoluchowski theory. The correction factor appears to have a value somewhere between 0.5 and 0.8. It is reasonable that this might arise from the neglect of particle-particle hydrodynamic interactions which become important when the particles are near (order of a particle diameter) each other.

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Polarographic Study of Iodothiophenes

By LEO WAYNE BROWN† and EDWARD KRUPSKI

Eight iodothiophenes were polarographed in two different solvents, N,N-dimethylformamide and 2-ethoxyethanol and the half-wave potential for each wave was calculated. The reduction of the iodothiophenes at the dropping mercury electrode was found to be essentially the same as reduction of the iodothiophenes by chemical methods. Reaction mixtures obtained in the preparation of some iodothiophenes were polarographed and the information obtained was found to be useful in determining the iodothiophenes present and their relative amounts. A study of the wave heights *vs.* concentration of 2-iodothiophene in N,N-dimethylformamide and in 2-ethoxyethanol was conducted and the diffusion current was shown to be proportional to the concentration.

THIOPHENE has been of interest in the field of organic synthesis because it possesses chemical characteristics similar to those of benzene and has been substituted for benzene and other aromatic nuclei in the synthesis of intermediates of pharmaceutical compounds and thiophene derivatives with therapeutic activity.

It was the intent of this project to make a polarographic study of iodothiophenes to provide additional information relative to the characteristics of the thiophene nucleus and to give an insight into the reactions taking place at the dropping mercury electrode.

Polarography has been used to compare the reactivities of the thiophene and benzene nucleus. Day and Blanchard (1) have studied the polarography of phenyl-2-thienyl ketone and 2,2′-dithienyl ketone in buffered solutions ranging from pH 1 to 13. Elving and Callahan (2) have studied the polarographic behavior of thenoyltrifluoroacetone and related compounds. Tirouflet and co-workers (3) have studied the polarographic behavior of thiophene derivatives and similarly substituted benzene derivatives. They compared 2-nitrothiophene, 2-iodothiophene, 2-thiophenealdehyde and other compounds with the corresponding benzene derivatives.

Since a direct correlation between the half-wave potential of corresponding benzene and thiophene derivatives had been shown, Tirouflet and Chane (4) studied the half-wave potential increments due to substituent X in the 5-position in 2- and 3-nitrothiophene and found them to be equal within experimental error with the corresponding increments in nitrobenzene with X-substituent in the *para* and *meta* position. The Hammett equation, which relates structure to both equilibrium constants and rate constants for the reactions of *meta*- and *para*-substituted benzene derivatives, was thus shown to hold for the thiophene series and sigma values were shown to be of the same order of magnitude. Further work by these same workers has shown that an extention of the Hammett equation to the thiophene series was possible in most cases, but must be carefully evaluated. Imoto and co-workers (5) have studied the effects of the benzene and thiophene nucleus on the polarographic half-wave potentials of the aromatic aldehydes and nitro compounds and concluded that Hammett's law is applicable to the half-wave potential of the thiophene derivatives.

EXPERIMENTAL

Polarographic Procedure.—A manual polarograph (Sargent polarograph model III) equipped with a standard mercury capillary electrode and a saturated calomel reference electrode in an H-type cell was used for the determinations. Nitrogen was passed through each solution of iodothiophene for 30 minutes to remove the oxygen from the solution before being polarographed.

All determinations were conducted in a constant temperature bath at 25 ± 0.5°. The current readings *vs.* voltage were plotted on graph paper and a smooth curve was drawn through the points. The half-wave potential was obtained from the graph by taking the mid-point of what appeared to be the most symmetrical curve.

The characteristics of the capillary in N,N-dimethylformamide with 0.05 M tetrabutylammonium iodide as the electrolyte were measured at 25 ± 0.5° with a mercury height of 60 cm. and are reported in Table I.

TABLE I.—CHARACTERISTICS OF CAPILLARY IN N,N-DIMETHYLFORMAMIDE

Volts	Time, sec.	m, mg./sec.
0.0	7.44	0.8588
1.0	6.86	0.8586
2.0	4.35	0.8680

The characteristics of the capillary in 2-ethoxyethanol with 0.05 M tetrabutylammonium iodide as

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the electrolyte were measured at 25 ± 0.5° with a mercury height of 60 cm. and are reported in Table II.

TABLE II.—CHARACTERISTICS OF CAPILLARY IN 2-ETHOXYETHANOL

Volts	Time, sec.	m, mg./sec.
0.0	7.39	0.8658
1.0	7.12	0.8626
2.0	4.67	0.8714

Purification of Solvents.—N,N-Dimethylformamide was dried over anhydrous potassium carbonate and distilled through a 90-cm. Fenske column. The fraction that distilled at 152–153° was collected.

Cellosolve (2-ethoxyethanol) was refluxed for 30 minutes with exsiccated ferrous sulfate and distilled through a 90-cm. Fenske column. The fraction that distilled at 135° was collected.

Tetrabutylammonium iodide was twice crystallized from ethyl acetate, suction filtered and 10-Gm. quantities placed in green glass containers. The containers were placed in a vacuum desiccator for 1 hour, then sealed and stored in a dark place.

Preparation of Iodothiophenes.—Tetraiodothiophene, 2-iodothiophene, 2,5-diiodothiophene, 3-iodothiophene, and 3,4-diiodothiophene were prepared essentially by the methods described in *Thiophene and its Derivatives* by Hartough (6). 2,3,4-Triiodothiophene, 2,3,5-triiodothiophene, and 2,3-diiodothiophene were prepared essentially by the methods described by Steinkopf (7).

Polarographic Results.—The eight iodothiophenes were polarographed in N,N-dimethylformamide and in 2-ethoxyethanol to obtain the half-wave potentials as shown in Tables III and IV.

The polarograms for all the prepared compounds, some mixtures of these compounds and several unknowns were plotted and analyzed but only a selected few of these polarograms are shown in this paper.

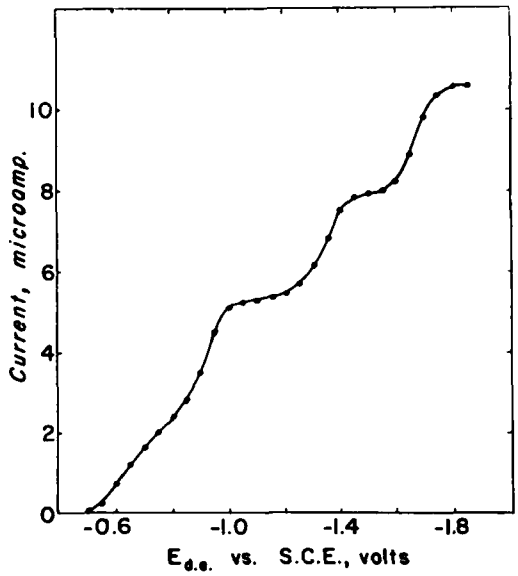

Fig. 1.—Polarogram of 1.0 millimolar tetraiodothiophene in N,N-dimethylformamide containing 0.05 *M* tetrabutylammonium iodide as electrolyte.

Four waves were obtained in the curve of the polarogram of tetraiodothiophene with the first two waves almost merging into one single large wave. The four waves were all of approximately the same height as shown in Fig. 1. 3-Iodothiophene, when polarographed, gave a single wave which corresponded to the last wave in tetraiodothiophene, consequently the last wave in tetraiodothiophene was judged to be caused by the iodine in the 3-position. To get a smooth wave from 3-iodothiophene, the concentration was maintained at about 0.5 millimolar because larger concentrations caused an inflection at the top of the wave as shown in Fig. 2. The polarogram of 2-iodothio-

TABLE III.—HALF-WAVE POTENTIALS IN N,N-DIMETHYLFORMAMIDE

Compound	Position of Iodine Causing Wave: 5	2	4	3
2-Iodothiophene	...	−1.21	...	...
2,3-Diiodothiophene	...	−0.89	...	−1.68
2,3,4-Triiodothiophene	...	−0.68	−1.32	−1.67
		−0.91		
2,3,4,5-Tetraiodothiophene	−0.67	−0.92	−1.34	−1.67
2,3,5-Triiodothiophene	−0.73	−0.97	...	−1.67
2,5-Diiodothiophene	−0.91	−1.23	...	...
3-Iodothiophene	...	...	...	−1.65
3,4-Diiodothiophene	...	...	−1.28	−1.66

TABLE IV.—HALF-WAVE POTENTIALS IN 2-ETHOXYETHANOL

Compound	Position of Iodine Causing Wave: 5	2	4	3
2-Iodothiophene	...	−1.16	...	...
2,3-Diiodothiophene	...	−0.88	...	−1.74
2,3,4-Triiodothiophene		−0.74	−1.27	−1.67
2,3,4,5-Tetraiodothiophene			Insoluble	
2,3,5-Triiodothiophene	−0.74	−0.93	...	−1.67
2,5-Diiodothiophene	−0.88	−1.21	...	...
3-Iodothiophene	...	...	...	−1.61
3,4-Diiodothiophene	...	...	−1.23	−1.67

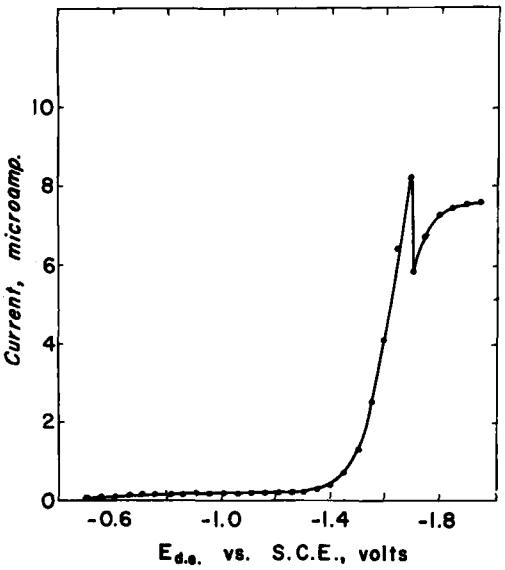

Fig. 2.—Polarogram of 1.78 millimolar 3-iodothiophene in 2-ethoxyethanol containing 0.05 *M* tetrabutylammonium iodide as electrolyte.

phene showed a single wave which corresponded with the second wave of tetraiodothiophene but it, too, gave an inflection when concentrations greater than 0.55 millimolar were used. Two maximum suppressors, Triton X-100 and methyl red, were added to the more concentrated solutions of 2-iodothiophene in an attempt to eliminate the inflections, but the suppressors had no effect on the inflections.

The curves of 2,3,5-triiodothiophene were similar to those of tetraiodothiophene except that the wave of the 4-position iodine was missing from the curves.

2,5-Diiodothiophene was polarographed to obtain a normal two-wave curve. In conjunction with this work a mixture of 2-iodothiophene and 2,5-diiodothiophene was polarographed and in the polarogram there was observed a short 5-position iodine wave and an extended 2-position iodine wave to indicate the ratio of these two substitutions. The polarograms of 3,4-diiodothiophene and 2,3-diiodothiophene were normal two-wave curves with the waves well distinguished from one another but with some shifting of the 2-position wave to a less negative potential in the case of the 2,3-diiodothiophene.

When 2,3,4-triiodothiophene was polarographed in N,N-dimethylformamide, four waves were produced but only the expected three waves were produced when polarographed in 2-ethoxyethanol. The sum of the wave heights of the first two waves of the curve of 2,3,4-triiodothiophene in N,N-dimethylformamide was equal to a single wave of the other two waves in the curve. Also, since only three waves were observed on the polarogram of 2,3,4-triiodothiophene in 2-ethoxyethanol, it was concluded that the first two waves of 2,3,4-triiodothiophene in N,N-dimethylformamide were caused by the single iodine in the 2-position. As the concentration of 2,3,4-triiodothiophene in N,N-dimethylformamide was decreased, the height of the first wave of the polarogram increased while the second wave decreased.

In one attempt to prepare 2,3,5-triiodothiophene by iodinating 3-iodothiophene in the 2 and 5 position, only an oil was obtained instead of solid 2,3,5-triiodothiophene. A polarogram was run with this oil and the curve in Fig. 3 was obtained. This curve showed that the reaction had not been run long enough since only a small amount of triiodothiophene was evident from the curve.

In the course of this study an experiment was conducted to determine the linearity of wave heights *vs.* concentration of 2-iodothiophene in N,N-dimethylformamide and in 2-ethoxyethanol. The results, shown in Fig. 4, indicated that the diffusion current was proportional to the concentration.

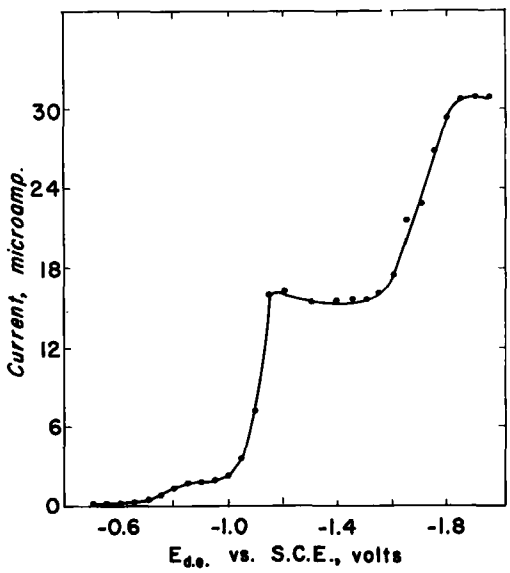

Fig. 3.—Polarogram of the oil obtained in preparing 2,3,5-triiodothiophene (3 drops per 50 ml. of N,N-dimethylformamide containing 0.5 *M* tetrabutylammonium iodide).

DISCUSSION

In order for data obtained from polarography to be of the utmost use to the chemist, a correlation between the normal chemical reaction and the reaction at the dropping mercury electrode should be obtained. Some researchers in this field suggest that the potential gradient near the surface of the mercury drop causes organic halides to approach the mercury surface with the halogen atom away from the drop and that this places the backside of the carbon holding the halogen in position to accept electrons, unless this is sterically interfered with by the presence of other groups. In addition, a large field effect should aid alkyl halide dissociation at all potentials. Lambert and Kobayashi (8) have studied the half-wave potentials of alkyl halides and cycloalkyl halides. They found good correlation between S_N2 reactivity and half-wave potential for many of these compounds which react by S_N2 reaction.

If the iodothiophenes approach the mercury surface with the iodine atoms away from the surface of the mercury, then it would seem that the polaro-

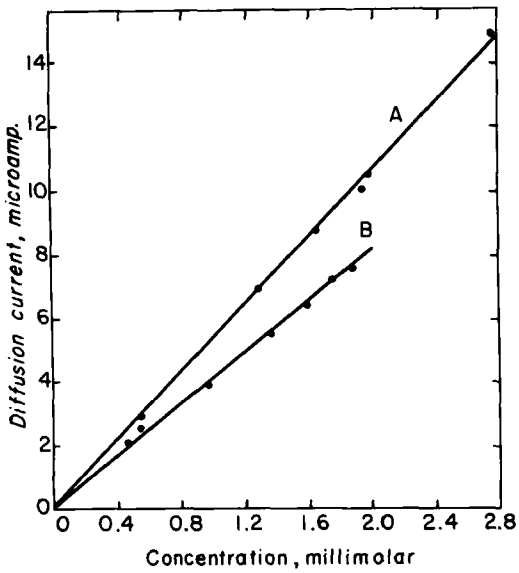

Fig. 4.—Plot of concentration *vs.* diffusion current of 2-iodothiophene in (*A*) N,N-dimethylformamide and (*B*) 2-ethoxyethanol.

gram of a compound such as 2,5-diiodothiophene would show a greater first wave height than second wave height since there are two identical carbons from which the removal of the first iodine atom can be made. In this study, the wave height produced by each iodine in a polyiodothiophene was approximately the same. Also, the position in which 2-iodothiophene approached the mercury surface would probably be different than the position in which 2,5-diiodothiophene approached the surface because they would have different dipole moments, yet in this study the half-wave potential of 2-iodothiophene corresponded fairly well with the 2-position iodine in 2,5-diiodothiophene. Levin and Fodiman (9) have shown earlier that a molecule such as the benzene nucleus containing *n* chlorine atoms was reduced at a potential independent of whether this molecule was introduced as such or was formed in the solution by reduction of a molecule richer in chlorine. It might be speculated then, in the case of the iodothiophenes, that the first electron could be added to the aromatic ring causing one of the ring electrons to shift to the iodine that is most easily reduced.

Marple, Hummelstedt, and Rogers (10) studied the polarographic reductions of benzyl halides. They proposed the following for the mechanism of reduction of benzyl halides at the dropping mercury electrode.

$$RX + e^- \rightarrow RX^- \quad \text{I}$$

$$RX^- \rightarrow R\cdot + X^- \quad \text{II}$$

$$R\cdot + e^- \rightarrow R^- \quad \text{III}$$

$$R^- + H_2O \rightarrow RH + OH^- \quad \text{IV}$$

$$2R\cdot \rightarrow R{-}R \quad \text{V}$$

$$RX + Hg \rightarrow RHgX \quad \text{VI}$$

It was generally assumed that addition of the first electron was the slow step and that reactions II, III, and IV were relatively rapid because only one wave was usually observed for each halogen atom replaced. Reactions V and VI were possible side reactions. They also found that benzyl iodide underwent two widely separated one-electron reduction steps at concentrations of less than 10^{-4} *M*. At higher concentrations, adsorption phenomena were encountered between the two waves due to the presence of bibenzyl, a by-product of the first reduction step. Judging from Marple and co-workers work, bithienyl might possibly have been formed as a side reaction and produced an adsorption wave causing the inflection observed in higher concentrations of 2-iodothiophene and 3-iodothiophene.

The behavior of 2,3,4-triiodothiophene in N,N-dimethylformamide in which a single iodine in the 2-position caused a double wave was probably an example of the kinetics of the reaction varying with the concentration.

The plot of concentration *vs.* diffusion current in Fig. 4 indicates that there exists a linear relationship between diffusion current and concentration of 2-iodothiophene in N,N-dimethylformamide and in 2-ethoxyethanol but that the diffusion current is higher in N,N-dimethylformamide than in 2-ethoxyethanol.

SUMMARY

Eight prepared iodothiophenes were reduced at a dropping mercury electrode of a manual polarograph in two different solvents, N,N-dimethylformamide and 2-ethoxyethanol, and their half-wave potentials were determined. The polarograms of the iodothiophenes in the two solvents were compared and discussed.

Polarograms were run on some of the reaction mixtures obtained in preparing the iodothiophenes in order to determine their composition. The information gained in these polarograms was of value in determining the length of time that the reaction should be run under the conditions used.

A study of the wave heights *vs.* concentration of 2-iodothiophene in N,N-dimethylformamide and in 2-ethoxyethanol was conducted and the diffusion current was shown to be proportional to the concentration; consequently a quantitative estimation of this compound could be obtained from its diffusion current.

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Stabilization of Vitamin B_{12} I

Complex Cyanides

By DONALD A. ZUCK and JAMES W. CONINE

The destruction of cyanocobalamin in liquid multiple vitamin preparations can be greatly reduced by the addition of small amounts of complex cyanides or iron salts. Cyanocobalamin stabilization with iron salts is not as effective as with complex cyanides under conditions designed to exclude air. The superiority of complex cyanides over iron salts was also demonstrated in partly filled containers where a large volume of air overlaid the product. The complex cyanides were also effective in reducing the destruction of cyanocobalamin induced by ultraviolet irradiation.

THE PROBLEM of deterioration of vitamin B_{12} in aqueous and multiple vitamin preparations was recognized shortly after its isolation as a pure crystalline compound (1, 2). Mention of stabilizers was first made in 1952 by Rosenblum (3) who reported that ferrous and ferric ions showed definite stabilizing properties on vitamin B_{12} in a multiple vitamin capsule. Additives such as sodium bisulfite (4), saccharated iron oxide (5), and thiodipropionic acid (6) found use as stabilizers with some degree of success.

Analogs of cyanocobalamin have been recognized (2, 11) as less stable forms. Other factors contributing to the instability of vitamin B_{12} in the complex preparations were air volume above the liquid and/or the area of the liquid-air interface (7), presence of copper ions with ascorbic acid (12), exposure to sunlight, direct ultraviolet light, or incandescent light (13).

The use of cyanide as a stabilizer was first mentioned in 1953 (8, 9). The process involved addition of hydrogen cyanide to the vitamin solution. Excess hydrogen cyanide was then removed by vacuum.

The question of vitamin B_{12} stability in multiple vitamin preparations has continued to be of major concern. Campbell (10) pointed out from a survey on marketed products that vitamin B_{12} was below label in all liquid and tablet formulations which were tested by his group.

Sodium pyrosulfite and sodium nitrite reduced vitamin B_{12} decomposition by 50% in liver preparations which were exposed to diffused light. This rapid decomposition of vitamin B_{12} in liver preparations, when exposed to air and light, was also reversed by the addition of sodium cyanide. Screenivasamurthy (14) found that light favored the conversion of the cyanocobalamin to the hydroxo form. The decomposition was then due to the instability of the hydroxo form in the presence of oxygen.

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More recently reported stabilizers were ammonium sulfite (15), iron compounds (16, 18, 19), organonitriles of the R—CH(OH)CN type (17), and metallocyanide complexes (20). This report deals with a comparative study of the iron salts and complex cyanide salts as stabilizers for vitamin B_{12} in a liquid multiple vitamin preparation in presence and absence of air. The effect of complex cyanides on the stability of an aqueous cyanocobalamin solution on exposure to ultraviolet light was also studied.

EXPERIMENTAL

A homogenized liquid multiple vitamin formulation was made up to 95% of the final volume of the preparation. Each milliliter of the finished preparation was to contain the following: vitamin A synthetic, 600 units; thiamine hydrochloride, 0.20 mg.; riboflavin, 0.24 mg.; pyridoxine hydrochloride, 0.20 mg.; nicotinamide, 2.0 mg.; ascorbic acid, 12.0 mg.; and vitamin D synthetic, 200 units.

The bulk preparation was stored under refrigeration. Cyanocobalamin, to give a final concentration of 1.0 mcg. per ml., and different stabilizers at predetermined amounts were dissolved in a minimum amount of purified water. This solution was then added to the bulk vitamin preparation which was then made up to 100% of volume with purified water. Such a multiple vitamin preparation was then filled under nitrogen cover into 4-oz. amber screw-capped bottles, and partly filled (40 oz.) under air cover into 1-gal. amber screw-capped bottles. These containers were stored at 26, 37, 46,[1] and 65° in constant temperature storage rooms or ovens. Assays were carried out by control laboratory using the U.S.P. microbiological method of assay for vitamin B_{12}.[2] The sampling schedule was based on results received in order to obtain the best distribution of data log-concentration-time plots.

For the ultraviolet irradiation study, solutions of 15 mcg. of cyanocobalamin containing potassium ferrocyanide 0.1 to 0.00005% and potassium ferricyanide 0.1 to 0.00001% were irradiated in a Fade-

[1] Upon completion of experiment it was found that the temperature in the 50° storage room was not uniform throughout. The area in which the bottles were stored was found to be 46°.

[2] The microbiological assays were performed by Mr. J. T. Stephenson and his associates of the Microbiological Testing Department, Eli Lilly and Co.

TABLE I.—EFFECT OF IRON COMPOUNDS ON STABILITY OF VITAMIN B_{12}

Stabilizer	Concn. Iron, mcg./ml.	Concn. Compd., mcg./ml.	% Initial at 1 yr. at 26° Nitrogen Cover Full Bottle	% Initial at 1 yr. at 26° Air Cover 1/4 Full Bottle	% Initial at 6 mo. at 37° Nitrogen Cover Full Bottle	% Initial at 6 mo. at 37° Air Cover 1/4 Full Bottle
None	0	0	10.5	21.4	31	<5
Peptonized iron	170	1000	88	56.5	82	41
Ferrous sulfate	170	850	77	51	67	29
Ferric ammonium citrate	170	1130	86	51	74	32
Ferrous gluconate	170	1460	76	50	75	35
Ferrous dihydrogen EDTA	170	1325	80	52.5	65	44
Ferric betaine citrate	170	1550	84	64	...	31
Potassium ferrocyanide	170	1320	98	100	90	81

TABLE II.—EFFECT OF CONCENTRATION OF IRON COMPOUNDS ON STABILITY OF VITAMIN B_{12}

Stabilizer	Concn. Iron, mcg./ml.	Concn. Compd., mcg./ml.	% Initial at 1 yr. at 26° Nitrogen Cover Full Bottle	% Initial at 1 yr. at 26° Air Cover 1/4 Full Bottle	% Initial at 6 mo. at 37° Nitrogen Cover Full Bottle	% Initial at 6 mo. at 37° Air Cover 1/4 Full Bottle
None	0	0	30	26	38	5
Ferrous gluconate	160	1370	79	50	86	38
Ferrous gluconate	120	1030	81	56	79	41
Ferrous gluconate	80	685	76	57	68	38
Ferrous gluconate	40	344	78	36	68	33
Potassium ferrocyanide	160	1250	93	89	95	77
Potassium ferrocyanide	120	937	91	94	95	74
Potassium ferrocyanide	80	625	96	96	97	84
Potassium ferrocyanide	40	312	87	91	95	79

Ometer[3] for periods of up to 96 hours at 52°. Samples were assayed over this period for vitamin B_{12} activity. Physical observations were also made and the pH was measured.

Reagents.—Cyanocobalamin concentrate B_{12} activity oral (3000 mcg. per Gm.) (Merck and Co.); vitamin B_{12} crystalline (Merck and Co.); iron peptonized N.F. IX powder; ferrous sulfate U.S.P.; ferric ammonium citrate N.F. XI; ferrous gluconate U.S.P.; ferrous dihydrogen ethylenediamine tetraacetic acid (Alrose Chemical Co.); ferric betaine citrate (Fleming Laboratories Inc.); potassium ferrocyanide reagent; potassium ferricyanide reagent; potassium cobalticyanide $K_3Co(CN)_6$ (Amend Drug & Chemical Co.); potassium manganocyanide $K_3Mn(CN)_6$ (Donald Axelson, U. of Ill.); potassium molybdocyanide $K_4Mo(CN)_8$ (Robert Novak, U. of Ill.); potassium nickelocyanide $K_2Ni(CN)_4 \cdot H_2O$ (City Chemical Corp.); potassium cuprocyanide $K_3Cu(CN)_4$ (City Chemical Corp.).

RESULTS AND DISCUSSION

Liquid Multiple Vitamin Product, Stabilization of Vitamin B_{12} in Presence of Vitamin C.—The results tabulated in Table I and Table II were from data obtained at the time and temperature indicated from full and partly filled containers for each of the stabilizers listed. Table I is a comparison of a number of stabilizers containing iron at 170 mcg. of iron per ml. Table II represents a study of ferrous gluconate and potassium ferrocyanide at four different concentrations. This was an attempt to determine the minimum effective concentration of stabilizer that could be used advantageously. Although the difference between the two stabilizers is quite striking, no appreciable change is observed in the effect of potassium ferrocyanide concentrations between 160 mcg. and 40 mcg. of iron per ml. Very little difference in stabilizing effect was noted with ferrous gluconate between the concentrations of 170 and 40 mcg. of iron per ml. It has been observed that potassium ferrocyanide and other metalocomplexes were effective at a metal-concentration as low as 10 mcg. per ml.

Because of amber glass containers which were used, light (13, 14) cannot be considered as a contributing factor in the conversion of the cyano-form to the relatively unstable hydroxo form in these experiments. It appears that a compound like potassium ferrocyanide, by furnishing cyanide ions, interferes with the transformation of the cyanocobalamin to the hydroxo or other less stable analogs of vitamin B_{12}. This action has been demonstrated in the study with ultraviolet irradiation. The analogs, once formed, were readily oxidized in presence of oxygen (14). A similar situation exists in the presence of ascorbic acid (1) where the cyano-form was found to be more stable than the other vitamin B_{12} analogs. Ferrous sulfate appears to inhibit the oxidation of vitamin B_{12}; however, this protection is reduced by an increase in air volume and/or surface area.

In Table III a comparison of six different metalocyanide complexes was made. The amounts added were such as to give 20 mcg. of metal per ml. of the vitamin preparation. There were no marked differences with respect to their stabilizing properties with the exception of $K_3Cu(CN)_4$. This complex exhibited a somewhat lesser stabilizing effect which became more evident in the partially filled containers at the higher temperatures. This behavior supports a previous observation (12) made with respect to copper ions, ascorbic acid, and vitamin B_{12}.

With the exception of $K_3Cu(CN)_4$ it would be difficult to select one of these complexes as a stabilizer for vitamin B_{12} on the basis of these results. But toxicity results show that potassium ferrocy-

[3] Atlas Electric Devices Co.

TABLE III.—EFFECT OF COMPLEX CYANIDES ON STABILITY OF VITAMIN B_{12}

Stabilizer	Concn. Iron, mcg./ml.	Concn. Compd., mcg./ml.	% Initial at 1 yr. at 26° Nitrogen Cover Full Bottle	% Initial at 1 yr. at 26° Air Cover 1/4 Full Bottle	% Initial at 6 mo. at 37° Nitrogen Cover Full Bottle	% Initial at 6 mo. at 37° Air Cover 1/4 Full Bottle
None	0	0	16	26	19	7
$K_3Co(CN)_6$	20	112	79	78	72	70
$K_2Ni(CN)_4$	20	88	84	68	77	48
$K_3Cu(CN)_4$	20	90	80	51	74	10
$K_3Mn(CN)_6$	20	119	94	72	79	66
$K_4Mo(CN)_8$	20	104	93	74	79	63
$K_4Fe(CN)_6$	20	150	93	81	81	72

TABLE IV.—DECOMPOSITION OF VITAMIN B_{12} FOLLOWING EXPOSURE TO ULTRAVIOLET IRRADIATION

$K_4Fe(CN)_6$, % Concn.	pH Initial	pH 8 hr.	% Loss 8 hr.	% Loss 8 to 96 hr.	% Loss Total	k, hr.$^{-1}$
0.1	6.4	8.1	97.3	1.8	99.1	...
0.02	6.45	8.6	84.0	2.0	86.0	0.00153
0.005	6.55	8.45	46.7	3.8	50.5	0.00107
0.001	6.15	6.70	7.8	14.4	22.2	0.00193
0.002	...	...	16.8	35.1	51.9	0.00416
0.00005	...	...	12.4	70.0	82.4	0.0182
0.00001	...	...	13.8	68.0	81.8	0.0172
0	5.82	5.90	14.5	75.5	90.0	0.0142

anide and potassium ferricyanide are the least toxic of the compounds studied with an LD_{50} oral in mice greater than 2 Gm. per Kg. Potassium cobalticyanide had an LD_{50} of 1529 ± 196 mg. per Kg. The LD_{50} of potassium molybdocyanide was found to be approximately 1500 mg. per Kg. Potassium nickelocyanide and potassium manganocyanide and potassium zinc cyanide had an LD_{50} of less than 275 mg. per Kg. This large variation in degree of toxicity is directly related to the ease with which the complex salts dissociate in solution (21). For example, the double nickelocyanide ($K_2[Ni(CN)_4]$) gives a precipitate with ammonium sulfide whereas potassium ferrocyanide gives no precipitate with either ammonium sulfide or with sodium hydroxide.

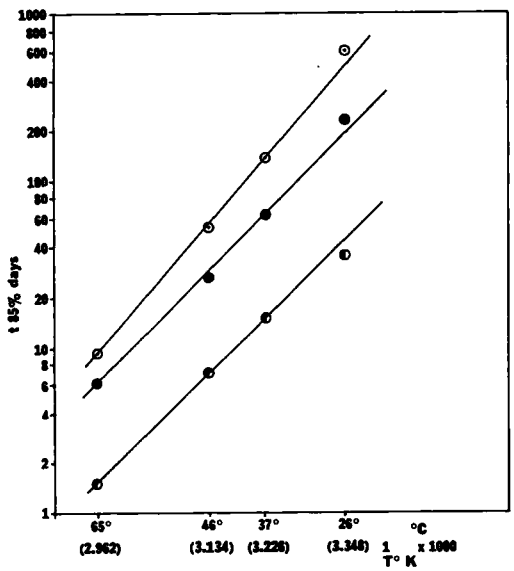

Fig. 1.—The relationship of $t_{85\%}$ (logarithmic scale) of vitamin B_{12} in a liquid multiple vitamin preparation and the reciprocal of the absolute temperature under nitrogen cover. ⊙, Potassium ferrocyanide; ●, ferrous gluconate; ◐, control.

The addition of iron at a concentration of 170 mcg. per ml. will stabilize vitamin B_{12} quite satisfactorily in both filled and partly filled containers. The superiority of potassium ferrocyanide as a stabilizer is not as pronounced in the full containers as it is in partly filled containers. This effect can be more readily seen in Fig. 1 and Fig. 2 in which the t_{85} (time for material to reach 85% of the initial concentration) was plotted against the reciprocal of the absolute temperature. The t_{85} values were obtained directly from the concentration-time plots at four different temperatures. Straight line plots indicated first-order reactions.[4]

There was no indication that changes in concentration of the stabilizer affected the decomposition rate for vitamin B_{12} between concentrations of potassium ferrocyanide at 20 to 170 mcg. of iron per ml. The t_{85} values for potassium ferrocyanide are averages of five separate experiments: 170, 40, 40, 20, and 20 mcg. of iron per ml. The t_{85} values representing ferrous gluconate are averages of three experiments: 170, 160, and 40 mcg. of iron per ml.

Figures 1 and 2 clearly demonstrate the superiority of potassium ferrocyanide over that of ferrous gluconate, particularly where contact with air is a factor. For example, under air cover, the time in days to reach 85% of initial at 26° was 320 days using potassium ferrocyanide *vs.* 90 days using ferrous gluconate (a ratio of 3.5:1.0); and under nitrogen cover the time was 560 days using potassium ferrocyanide *vs.* 230 days with ferrous gluconate (a ratio of 2.4:1.0).

Cyanocobalamin Solution, Effect of Cyanides on Decomposition Due to Ultraviolet Irradiation.—Results obtained from the ultraviolet degradation studies are tabulated in Tables IV and V.

A rapid destruction of vitamin B_{12} was observed at the higher concentrations of the complex. Over the limits of this particular study this decomposition

[4] The assay at 1 month for the control experiment at 26° in partly filled containers showed a greater drop than was expected. Thereafter, a first-order decomposition followed.

TABLE V.—DECOMPOSITION OF VITAMIN B_{12} FOLLOWING EXPOSURE TO ULTRAVIOLET IRRADIATION

$K_4Fe(CN)_6$, % Concn.	pH		% Loss			k, hr.$^{-1}$
	Initial	8 hr.	8 hr.	8 to 96 hr.	Total	
0.1	6.75	9.6	92.0	6.3[a]	98.3	...
0.02	6.7	9.3	48.3	6.7	55.0	0.00157
0.005	6.85	8.95	18.7	7.8	26.5	0.00115
0.001	6.40	6.9	16.3	9.8	26.1	0.00143
0.0002	...	...	21.4	47.7	69.1	0.0106
0.00005	...	...	19.5	67.6	87.1	0.0208
0	...	...	13.9	78.5	92.4	0.0405

[a] Extrapolated value.

was proportional to the amount of cyanide added. The initial reaction appeared to be destruction of the vitamin B_{12} activity. After this, a stabilizing effect on the remaining vitamin B_{12} activity was observed. The optimum concentration for potassium ferricyanide was 0.001% and for potassium ferrocyanide it was from 0.001 to 0.005%. The stabilizing effect decreased rapidly below these concentrations. The optimum stabilizing effect of potassium cyanide under the same conditions was 0.001% (25). With concentrations greater than this, there was no improvement in the stabilizing effect. Tables IV and V give the per cent of initial concentration lost in the first 8 hours and the per cent of initial concentration lost in the remaining 88 hours. The total per cent loss is also given. Figure 3 illustrates the type of curve obtained when the logarithm of per cent of the initial vitamin B_{12} concentration was plotted against time. After 8 hours' exposure, a precipitate giving a positive test for iron was formed, the pH changed as listed in Tables IV and V, and a pronounced odor of cyanide was present in solutions.

At this time the reasons that the two complexes accelerate the degradation of vitamin B_{12} during their photochemical decomposition are not clear.

The photochemical degradation of potassium ferrocyanide has been studied previously (22–24). Although our work was done on less concentrated solutions, it confirms the previously reported results.

The reaction for potassium ferrocyanide in presence of air is believed to be

$$2Fe^{++} + 4OH^- + H_2O + {}^1/_2O_2 \rightarrow 2Fe(OH)_3$$

In the absence of air the reaction is

$$Fe(CN)_6^{----} + HOH \rightleftharpoons Fe(CN)_5 \cdot H_2O^{---} + CN^-$$

and then followed by

$$CN^- + HOH \rightleftharpoons HCN + OH^-$$

Since the glass-sealed ampuls had a limited supply of air, both reactions could have occurred.

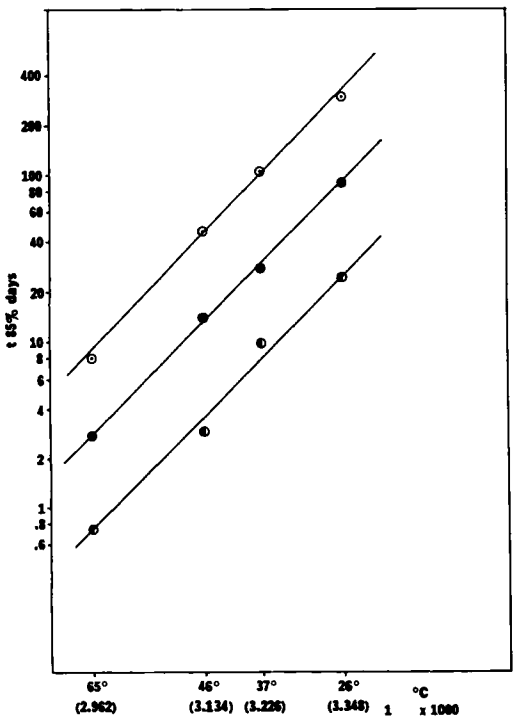

Fig. 2.—The relationship of $t_{85\%}$ (logarithmic scale) of vitamin B_{12} in a liquid multiple vitamin preparation and the reciprocal of the absolute temperature in presence of air. ⊙, Potassium ferrocyanide; ●, ferrous gluconate; ◐, control.

SUMMARY

1. A comparative study of the stabilizing effect of ferrous salts and complex cyanides on

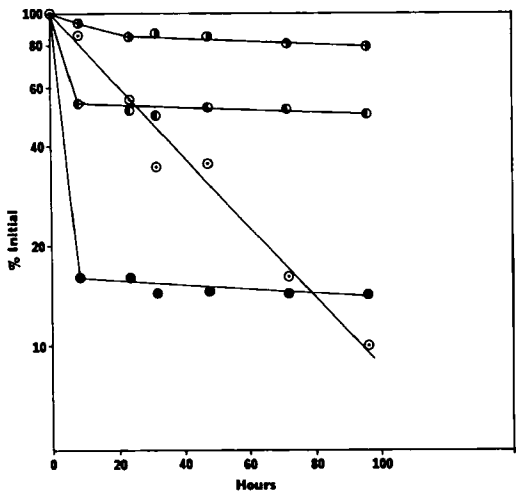

Fig. 3.—Decomposition of vitamin B_{12} in the presence of potassium ferricyanide following exposure to ultraviolet irradiation in the Fade-Ometer. ●, 0.02% potassium ferricyanide; ◐, 0.005% potassium ferricyanide; ◑, 0.001% potassium ferricyanide; ⊙, control.

vitamin B_{12} in multiple vitamin preparations in the presence and absence of air was made.

2. The superiority of potassium ferrocyanide over iron salts as a vitamin B_{12} stabilizer in multiple vitamin preparations was demonstrated.

3. The destruction of vitamin B_{12} by ultraviolet light was investigated. Under the conditions of the experiment it was found that the optimum stabilizing concentration of potassium ferrocyanide and potassium ferricyanide was 0.001 to 0.005% and 0.001%, respectively.

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Stabilization of Vitamin B_{12} II

α-Hydroxynitriles

By JAMES W. CONINE and DONALD A. ZUCK

The destruction of vitamin B_{12} in liquid multiple vitamin products can be greatly reduced by the addition of α-hydroxynitriles or their esters. The stabilizing effect appears to be due to decomposition of the α-hydroxynitrile into hydrogen cyanide and the corresponding aldehyde. An initial stabilization of cyanocobalamin was seen in solutions which contained α-hydroxynitriles and were exposed to ultraviolet irradiation. However, upon long exposure to ultraviolet light, cyanocobalamin solutions containing α-hydroxynitriles eventually decomposed more than the control.

VITAMIN B_{12} has been shown to be extremely susceptible to decomposition in the presence of ascorbic acid (1, 2). A recent review (3) has covered a number of compounds which have been suggested as stabilizers to reduce the rate of decomposition of vitamin B_{12}. Some of these, such as nitrites or bisulfites, are not completely satisfactory because they have an adverse affect upon the stability of thiamine or some of the other vitamins. Hydrogen cyanide has been used successfully to stabilize vitamin B_{12} injections (4, 5), and cyanides have been utilized in the production of cyanocobalamin (6). α-Hydroxynitriles and their esters are potential sources of cyanide and as such have been shown to be effective stabilizers of vitamin B_{12} in pharmaceutical preparations (7). The effect of α-hydroxynitriles and their esters upon the rate of decomposition of vitamin B_{12} is the subject of the present study.

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EXPERIMENTAL

Liquid Multiple Vitamin Preparation.—A homogenized liquid multiple vitamin formulation was made up to contain 95% of the final volume of the preparation. Each milliliter of the finished preparation was to contain: vitamin A, 600 units; vitamin B_1, 0.2 mg.; vitamin B_2, 0.24 mg.; vitamin B_6, 0.2 mg.; nicotinamide, 2.0 mg.; ascorbic acid, 12 mg.; and vitamin D synthetic, 200 units. To this preparation was added a triturate of crystalline cyanocobalamin in mannitol to give the preparation a concentration of 1 mcg. per ml. of vitamin B_{12}. The α-hydroxynitrile was dissolved in distilled water or aqueous alcohol and added in the appropriate concentration. The product was brought up to final volume with distilled water and stirred until uniform. The samples were filled into amber

vitamin B_{12} in multiple vitamin preparations in the presence and absence of air was made.

2. The superiority of potassium ferrocyanide over iron salts as a vitamin B_{12} stabilizer in multiple vitamin preparations was demonstrated.

3. The destruction of vitamin B_{12} by ultraviolet light was investigated. Under the conditions of the experiment it was found that the optimum stabilizing concentration of potassium ferrocyanide and potassium ferricyanide was 0.001 to 0.005% and 0.001%, respectively.

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Stabilization of Vitamin B_{12} II

α-Hydroxynitriles

By JAMES W. CONINE and DONALD A. ZUCK

The destruction of vitamin B_{12} in liquid multiple vitamin products can be greatly reduced by the addition of α-hydroxynitriles or their esters. The stabilizing effect appears to be due to decomposition of the α-hydroxynitrile into hydrogen cyanide and the corresponding aldehyde. An initial stabilization of cyanocobalamin was seen in solutions which contained α-hydroxynitriles and were exposed to ultraviolet irradiation. However, upon long exposure to ultraviolet light, cyanocobalamin solutions containing α-hydroxynitriles eventually decomposed more than the control.

VITAMIN B_{12} has been shown to be extremely susceptible to decomposition in the presence of ascorbic acid (1, 2). A recent review (3) has covered a number of compounds which have been suggested as stabilizers to reduce the rate of decomposition of vitamin B_{12}. Some of these, such as nitrites or bisulfites, are not completely satisfactory because they have an adverse affect upon the stability of thiamine or some of the other vitamins. Hydrogen cyanide has been used successfully to stabilize vitamin B_{12} injections (4, 5), and cyanides have been utilized in the production of cyanocobalamin (6). α-Hydroxynitriles and their esters are potential sources of cyanide and as such have been shown to be effective stabilizers of vitamin B_{12} in pharmaceutical preparations (7). The effect of α-hydroxynitriles and their esters upon the rate of decomposition of vitamin B_{12} is the subject of the present study.

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EXPERIMENTAL

Liquid Multiple Vitamin Preparation.—A homogenized liquid multiple vitamin formulation was made up to contain 95% of the final volume of the preparation. Each milliliter of the finished preparation was to contain: vitamin A, 600 units; vitamin B_1, 0.2 mg.; vitamin B_2, 0.24 mg.; vitamin B_6, 0.2 mg.; nicotinamide, 2.0 mg.; ascorbic acid, 12 mg.; and vitamin D synthetic, 200 units. To this preparation was added a triturate of crystalline cyanocobalamin in mannitol to give the preparation a concentration of 1 mcg. per ml. of vitamin B_{12}. The α-hydroxynitrile was dissolved in distilled water or aqueous alcohol and added in the appropriate concentration. The product was brought up to final volume with distilled water and stirred until uniform. The samples were filled into amber

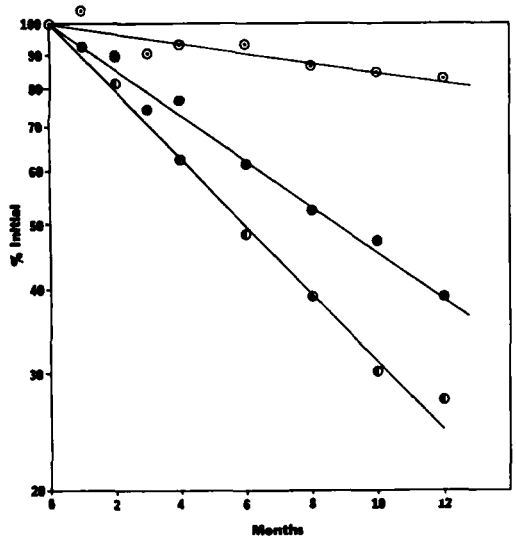

Fig. 1.—First-order decomposition of vitamin B_{12} at 26° in a liquid multiple vitamin preparation containing esters of mandelonitrile. ⊙, 0.1% acetylmandelonitrile; ●, 0.1% benzoylmandelonitrile; ◐, control.

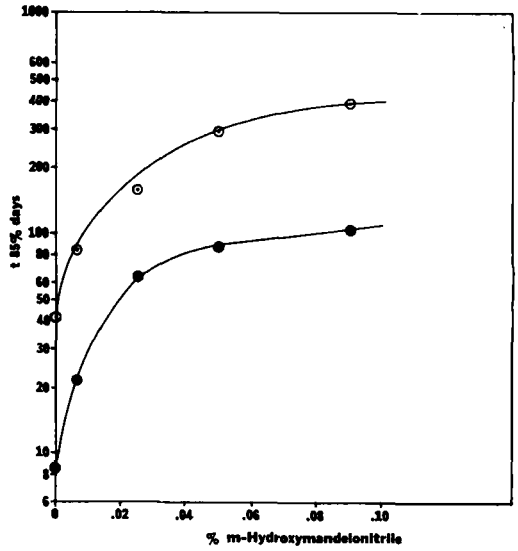

Fig. 2.—The relationship of $t_{85\%}$ of vitamin B_{12} in a liquid multiple vitamin preparation (logarithmic scale) and concentration of *m*-hydroxymandelonitrile. ⊙, at 26°; ● , at 37°.

1-pt. bottles and stored at 26 and 37° in controlled temperature rooms. Samples were removed periodically and assayed for vitamin B_{12} activity by the microbiological method of the U.S.P.[1]

Solution Cyanocobalamin.—Solutions of cyanocobalamin, 15 mcg. per ml., were prepared. Before the solution was made up to final volume the α-hydroxynitriles were added from a stock solution. Water was used to dissolve *m*-hydroxymandelonitrile. Ethanol, 95%, was used as a solvent for carbethoxymandelonitrile, and the solutions in this series were prepared at a final concentration of 5% ethanol. The solutions were filled into 5-ml. No. 1 flint glass ampuls and irradiated in the Fade-Ometer[2] at a temperature of 52° for periods of up to 96 hours. The ampuls were assayed for vitamin B_{12} activity by microbiological assay method of the U.S.P.[1]

The substituted mandelonitriles were prepared by the method of Buck (8). The esters of mandelonitrile were prepared by the method of Francis and Davis (9). Lactonitrile,[3] amygdalin, and aniline-free benzonitrile were also used in this study.

DISCUSSION AND RESULTS

The effect of five α-hydroxynitriles and four esters of mandelonitrile upon the stability of vitamin B_{12} in a liquid multiple vitamin preparation was studied and the results are listed in Table I.

The decomposition of vitamin B_{12} follows first-order kinetics as indicated by the straight line relationship obtained when the concentration of vitamin B_{12} was plotted on a logarithmic scale against the age of the sample (Fig. 1). In order to determine the effect of concentration of α-hydroxynitrile upon the rate of vitamin B_{12} decomposition, several concentrations of *m*-hydroxymandelonitrile were studied and these results are listed in Table II.

TABLE I.—PER CENT OF VITAMIN B_{12} REMAINING AFTER AGING SAMPLE

Preparation, 0.1%	1 yr. at 26°, %	6 mo. at 37°, %
Control	21.7	3.2
Benzoylmandelonitrile	39	10.8
Carbethoxymandelonitrile	76	67.5
Benzene sulfonyl mandelonitrile	76.6	68
Acetylmandelonitrile	82	85
Lactonitrile	89	76
p-Methoxymandelonitrile	79	77
o-Chloromandelonitrile	89	84.5
m-Hydroxymandelonitrile	87	80
3-Methoxy-4-hydroxymandelonitrile	90.3	81

TABLE II.—EFFECT OF CONCENTRATION OF *m*-HYDROXYMANDELONITRILE ON STABILITY OF VITAMIN B_{12}

	Vitamin B_{12} % Initial Assay, 12 mo. at 26°	k, day^{-1}	Vitamin B_{12} % Initial Assay, 6 mo. at 37°	k, day^{-1}
Control	27.2	0.00392	3.2	0.0188
m-Hydroxymandelonitrile, %				
0.1	89	0.00043	77.3	0.00159
0.05	88.6	0.000575	71.5	0.00189
0.025	90.2	0.00108[a]	67	0.00255
0.00625	67	0.00201	34.1	0.00724

[1] The microbiological assays were performed by Mr. J. T. Stephenson and his associates of the Microbiological Testing Department, Eli Lilly and Co.

[2] Atlas Electric Devices Co.

[3] American Cyanamid Co.

[a] The plot of the log concentration *vs.* storage time changes from one rate to another at 10–12 months; the more rapid reaction rate is given here.

Figure 2 is a graph of $t_{85\%}$ (the time required for vitamin B_{12} concentration to reach 85% of the initial assay value) plotted on a logarithmic scale against the concentration of *m*-hydroxymandelonitrile. The $t_{85\%}$ was calculated from *k* values obtained by drawing the best straight line through the points obtained by plotting log concentration of vitamin B_{12} against the sample age in days. The most rapid change in reaction rate occurred at the lowest concentrations of nitrile. As the amount of of *m*-hydroxymandelonitrile was increased up to 0.1%, successively less change was produced for each unit of α-hydroxynitrile added. This indicates that the reaction rate may be related to the equilibrium reaction between two forms of vitamin B_{12}, cyanocobalamin and a noncyano form.

$$\text{cyanocobalamin} \rightleftharpoons \text{noncyanocobalamin} + [CN^-]$$

$$k = \frac{[\text{noncyanocobalamin}]\,[CN^-]}{\text{cyanocobalamin}}$$

It has been reported that at pH 2.5–3.0 at room temperature pure cyanocobalamin decomposes at the rate of about 1.5% a day in the presence of ascorbic acid, compared with almost complete destruction of hydroxocobalamin (vitamin B_{12a}) in 1 day under similar conditions (2). In the presence of cyanide the equilibrium is shifted toward the more stable cyanocobalamin and away from the less stable compound.

Equilibrium constants have been reported for the dissociation of α-hydroxynitriles (10). These compounds dissociate to a greater extent in aqueous solutions than when water is absent (11). If the dissociation is sufficiently in favor of the formation of cyanide, this in turn will favor the formation of cyanocobalamin

$$\begin{array}{l} \text{RCH} - \text{CN} \rightleftharpoons \text{RCHO} + \text{HCN} \\ \quad\;\; | \qquad\qquad\qquad\quad \Uparrow \\ \quad\;\; \text{OH} \qquad\qquad\qquad\quad \Downarrow \end{array}$$

$$\text{cyanocobalamin} \rightleftharpoons \text{noncyanocobalamin} + \text{HCN}$$

All the compounds employed in this study demonstrated this stabilizing effect on vitamin B_{12} except amygdalin and benzonitrile. Amygdalin would be expected to undergo dissociation more slowly than either free α-hydroxynitriles or the esters. Benzonitrile should not dissociate to form cyanide. Benzoyl mandelonitrile was not as satisfactory a stabilizer as the other esters or free nitriles, possibly because of a slower rate of hydrolysis.

Aqueous cyanocobalamin solutions are stable for long periods of time under ordinary storage conditions, but decompose readily upon exposure to ultraviolet light (12). Solutions which contained 15 mcg. per ml. of cyanocobalamin and from 0.1 to 0.001% of *m*-hydroxymandelonitrile were exposed to ultraviolet irradiation on the Fade-Ometer for periods of up to 96 hours. The results were plotted in Fig. 3 as log per cent of the initial assay against time of exposure.

The rate of decomposition of cyanocobalamin in the presence of α-hydroxynitrile did not follow first-order kinetics as did the control sample. Plots of the decomposition of cyanocobalamin in the

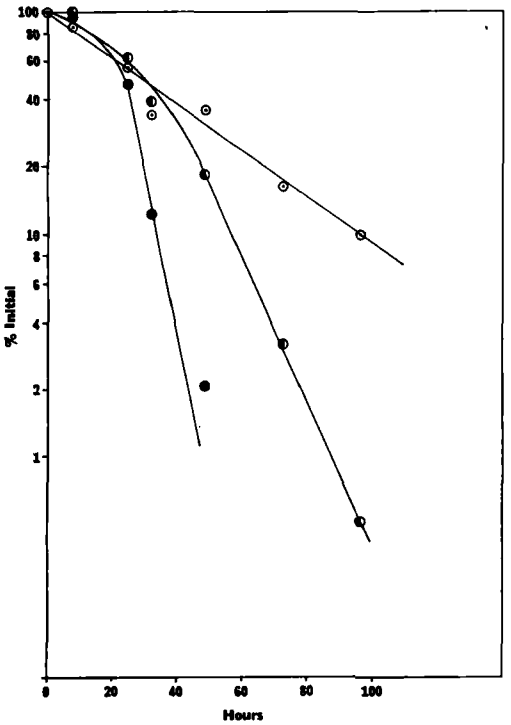

Fig. 3.—Decomposition of vitamin B_{12} in the presence of *m*-hydroxymandelonitrile following exposure to ultraviolet irradiation in the Fade-Ometer. ●, 0.1% *m*-hydroxymandelonitrile; ◐, 0.001% *m*-hydroxymandelonitrile; ⊙, control.

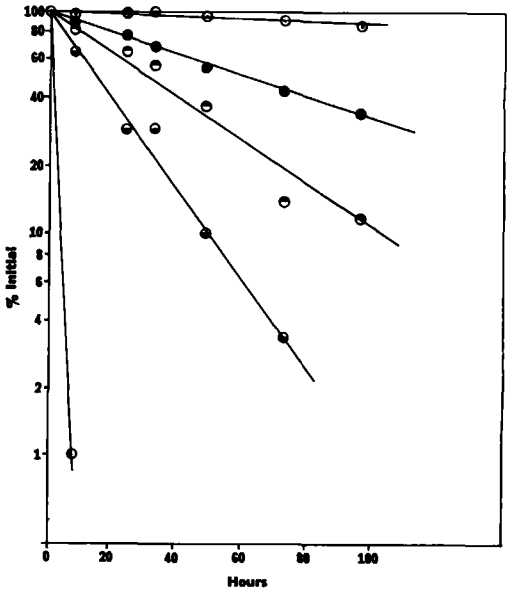

Fig. 4.—Decomposition of vitamin B_{12} in the presence of potassium cyanide or *m*-hydroxymandelonitrile following exposure to ultraviolet irradiation in the Fade-Ometer. ⊙, 0.001% potassium cyanide; ●, 0.0002% potassium cyanide; ◐, 0.1% *m*-hydroxybenzaldehyde; ◒, 0.001% *m*-hydroxybenzaldehyde; ◓, control.

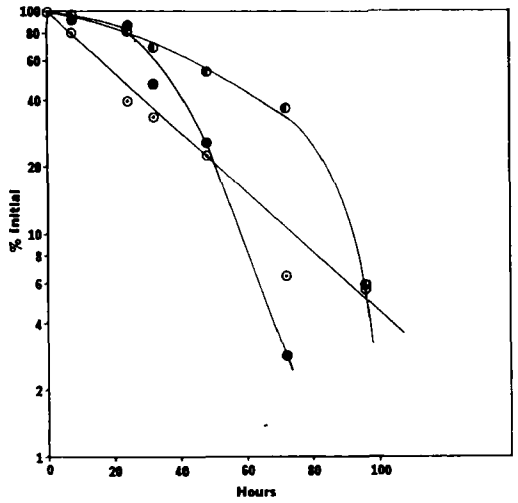

Fig. 5.—Decomposition of vitamin B_{12} in the presence of carbethoxymandelonitrile following exposure to ultraviolet irradiation in the Fade-Ometer. ●, 0.1% carbethoxymandelonitrile; ◐, 0.005% carbethoxymandelonitrile; ⊙, control.

presence of potassium cyanide and *m*-hydroxybenzaldehyde are illustrated in Fig. 4. The reactions in both of these cases follow first-order kinetics. The fact that solutions containing *m*-hydroxymandelonitrile do not follow first-order kinetics suggests that the rate at which equilibrium is reached in the dissociation of the nitrile to the aldehyde and hydrogen cyanide is the factor controlling the rate of decomposition of cyanocobalamin. In the cases where carbethoxymandelonitrile was used, the change in reaction rate was much slower (Fig. 5). This would be expected since hydrolysis of the ester precedes the dissociation of the mandelonitrile. The hydrolysis rate is the rate-controlling step in this reaction. The eventual reaction rate at which vitamin B_{12} decomposes should be that for the equilibrium mixture of aldehyde, hydrogen cyanide and α-hydroxynitrile.

Cyanocobalamin solutions containing α-hydroxynitriles after short periods of irradiation are more stable than the control solution of cyanocobalamin alone. After longer periods of irradiation they are less stable than the control. One explanation for this is that a competition exists between the cobalamin and the aldehyde for the cyanide in solution and that the ratio of cobalamin to free aldehyde is an important factor in the loss of vitamin B_{12} activity. If the effect of cyanide and aldehyde upon the rate of decomposition of cyanocobalamin were independent, the change from a rate slower than the control to one which is faster would not be expected to occur. Cyanide salts are much more effective in stabilizing cyanocobalamin in the presence of ultraviolet light than is cyanide in the form of an α-hydroxynitrile.

Acute oral toxicity in the mouse was obtained for two compounds (Table III). Although these

TABLE III.—ACUTE ORAL TOXICITY OF α-HYDROXYNITRILES IN MICE

	LD_{50}, mg. per Kg.
m-Hydroxymandelonitrile	25.40 ± 1.47
Carbethoxymandelonitrile	34.86 ± 2.34

compounds have a low LD_{50}, they may still be useful because of the low concentration required for vitamin B_{12} stabilization. The optimum concentration for *m*-hydroxynitrile based on both efficacy and toxicity is close to 0.05%. No toxicity studies have been made on pharmaceutical preparations.

SUMMARY

1. α-Hydroxynitriles and esters of mandelonitrile were shown to be effective stabilizers of vitamin B_{12} in a liquid multiple vitamin preparation.

2. *m*-Hydroxymandelonitrile and carbethoxymandelonitrile demonstrated some stabilizing action on vitamin B_{12} solutions which were exposed to ultraviolet irradiation. The initial stabilizing effect is reversed upon longer exposure and, at or before 96 hours, all the samples containing α-hydroxynitriles were decomposing at a greater rate than the control samples.

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Dissolution Rates of Finely Divided Drug Powders I

Effect of a Distribution of Particle Sizes in a Diffusion-Controlled Process

By W. I. HIGUCHI† and E. N. HIESTAND

An equation is derived to describe the dissolution rate of a particle with time in a diffusion-controlled dissolution process. This is applied to a hypothetical powder whose particles are approximately log-normally distributed. The change in size distribution with time is shown. Also, the dissolution rate of such a powder is compared with that of a monosized powder whose radius equals the mass median radius of the powder.

THE dissolution rate of many finely divided powders is diffusion controlled. In addition to the diffusion rate constant, the effect of the size distribution must be included in order to calculate the dissolution rate. This paper discusses the use of equations suitable for making these calculations.

THEORY

The Dissolution of a Single Particle.—Several simplifying assumptions enable one to describe the dissolution of a single particle. These assumptions are: (*a*) The dissolution rate is diffusion controlled. (*b*) The diffusion layer thickness is always the same for all particles of the same size and is comparable to or greater than the particle radius. (*c*) The concentration change of dissolved solid in the bulk solution is negligible at all times. (*d*) The effective particle shape approximates a sphere.

From Fick's law of diffusion,

$$\frac{dm}{dt} = D4\pi r^2 \frac{dC}{dr} \qquad \text{(Eq. 1)}$$

where m is the weight of a solid particle, D is the diffusion rate constant, C is the concentration of dissolved solid, t is time, and r is the radius of an imaginary sphere through which the diffusion occurs.

If a is the radius of the particle at time t, then $r > a$. The second assumption, (*b*), implies a quasi-steady state condition in the diffusion layer. Hence, the time required to establish this steady state condition is neglected (see the Appendix). Therefore, one may write

$$\frac{dm}{dt}\int_a^\infty \frac{dr}{r^2} = 4\pi D \int_{C_s}^{C_o} dC$$

C_s is the saturation concentration and C_o is the concentration in the bulk of the solution. Upon integration one obtains

$$\frac{dm}{dt} = -4\pi D a \Delta C \qquad \text{(Eq. 2)}$$

where $\Delta C = C_s - C_o$.

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Since it has been assumed that ΔC is independent of time, particle size, a, is the only variable with time. From the volume of the particle, v, and density, ρ

$$\frac{dm}{dt} = \frac{d(v\rho)}{dt} = 4\pi\rho a^2 \frac{da}{dt} \qquad \text{(Eq. 3)}$$

Equating 2 and 3

$$a\rho \frac{da}{dt} = -D\Delta C$$

Let $a = a_o$ at $t = o$, then

$$\int_{a_o}^{a} a\, da = -\frac{D\Delta C}{\rho}\int_o^t dt$$

$$a^2 = a_o^2 - \frac{2D\Delta C t}{\rho} \qquad \text{(Eq. 4)}$$

Equation 4 provides a means of calculating any particle diameter at time t if its radius at zero time is known.

The Total Dissolution Rate.—Let the particle size distribution at zero time be characterized by some function of a_o

$$n = n(a_o) \qquad \text{(Eq. 5)}$$

then the total number of particles, N, in any increment of sizes is

$$N = \int_{a_{so}}^{a_{lo}} n(a_o)\, da_o \qquad \text{(Eq. 6)}$$

where a_{lo} and a_{so} are the largest and smallest particles at zero time in the increment; and by defining a_{so} and a_{lo} as the extremes of the size distribution, N becomes the total number of particles in the entire sample.

The total mass of undissolved drug at

$$t \ll \frac{a_{so}^2 \rho}{2D\Delta C}$$

is

$$M = \int_{a_{so}}^{a_{lo}} 4/3\pi\rho a^3 n(a_o)\, da_o \qquad \text{(Eq. 7)}$$

where a in the integral is given by Eq. 4.

When

$$t > \frac{a_{so}^2 \rho}{2D\Delta C}$$

the lower limit of integration must be changed to correspond to the zero time radius of the particle that has just dissolved at time t, *viz.* a_{ot}

$$a_{ot} = \left(\frac{2D\Delta Ct}{\rho}\right)^{1/2} \qquad \text{(Eq. 8)}$$

Combining Eqs. 4 and 7, and using the lower limit defined in Eq. 8, the amount of undissolved drug at time t is

$$M = \int_{a_{ot}}^{a_{lo}} 4/3\pi\rho\left(a_o^2 - \frac{2D\Delta Ct}{\rho}\right)^{3/2} n(a_o)\, da_o \qquad \text{(Eq. 9)}$$

so the fraction undissolved, Q, at time t is

$$Q = \frac{\int_{a_{ot}}^{a_{lo}} 4/3\pi\rho\left(a_o^2 - \frac{2D\Delta Ct}{\rho}\right)^{3/2} n(a_o)\, da_o}{M_o} \qquad \text{(Eq. 10)}$$

or

$$Q = \frac{\int_{a_{ot}}^{a_{lo}} \left(a_o^2 - \frac{2D\Delta Ct}{\rho}\right)^{3/2} n(a_o)\, da_o}{\int_{a_{so}}^{a_{lo}} a_o^3 n(a_o)\, da_o}$$

where M_o is the total weight of solid at zero time.

The Cumulative Distribution Curve.—The use of the cumulative plot of per cent of sample above a given size *vs.* the size is most convenient, especially when the particles are approximately log-normally distributed as they usually are when produced by milling. The fraction larger than a_{io} is given by

$$f > a_{io} = \frac{\int_{a_{io}}^{a_{lo}} 4/3\pi\rho a_o^2 n(a_o)\, da_o}{M_o} \qquad \text{(Eq. 11)}$$

The Change in Size Distribution with Time.—It is possible to evaluate the size distribution at times after the dissolution has started. When

$$t \leqslant \frac{a_{so}^2\rho}{2D\Delta C}$$

$$f > a_i = \frac{\int_{a_{io}}^{a_{lo}} \left(a_o^2 - \frac{2D\Delta Ct}{\rho}\right)^{3/2} n(a_o)\, da_o}{\int_{a_{so}}^{a_{lo}} \left(a_o^2 - \frac{2D\Delta Ct}{\rho}\right)^{3/2} n(a_o) da_o} \qquad \text{(Eq. 12)}$$

or when

$$t > \frac{a_{so}^2\rho}{2D\Delta C}$$

$$> a_i = \frac{\int_{a_{io}}^{a_{lo}} \left(a_o^2 - \frac{2D\Delta Ct}{\rho}\right)^{3/2} n(a_o)\, da_o}{\int_{a_{ot}}^{a_{lo}} \left(a_o^2 - \frac{2D\Delta Ct}{\rho}\right)^{3/2} n(a_o) da_o} \qquad \text{(Eq. 13)}$$

Obviously the integrals in Eqs. 12 and 13 are the same form as in Eq. 9 and may be evaluated if Eq. 9 may be evaluated.

The Size Distribution Function.—Equation 5 states that the size distribution is some function of a_o. Equation 9 is sufficiently complex that the integration becomes difficult if the distribution function is not simple. In practice, many materials have particle size distributions that correspond to a relatively simple function of a_o. Such a case will be described in a later publication which gives a test of the theory presented here.

Because many materials when milled produce a size distribution that approximates a log-normal distribution, it would be useful to substitute for $n(a_o)$ the log-normal distribution function. However, this becomes a somewhat complex case.

A log-normal distribution when plotted as n *vs.* a_o gives a skewed curve with the long tail toward the large diameters. In such a case the mass median diameter is much larger than the diameter corresponding to the mode of the histogram. Consequently, most of the mass is present in the region of the distribution where n is increasing as a_o is decreasing. Therefore, distributions approximately log-normal may also be approximately characterized over the region accounting for most of the mass by some inverse power of the radius. Figure 1 shows the reasonable correlation of such a case. This case corresponds to a distribution according to the equation

$$\frac{nv_o}{V_o} = \frac{K'}{a_o} \qquad \text{(Eq. 14)}$$

where $v_o = 4/3\ \pi\ a_o^3$; $V_o = \sum_{a_{so}}^{a_{lo}} nv_o$ and K' is a constant. Hence

$$n = \frac{K}{a_o^4} \qquad \text{(Eq. 15)}$$

where K is another constant.

Note that Fig. 1 was plotted on log-probability paper; a straight line represents a material that is log-normally distributed. A plot of values cal-

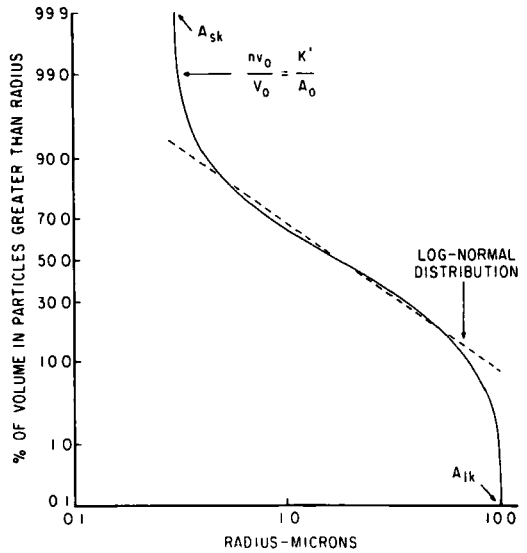

Fig. 1.—Comparison of the log-normal distribution with a simpler case, $n = \frac{K}{a_o^4}$.

culated using Eqs. 1 and 15 fits the log-normal line very closely except for the upper and lower 10% by volume. The contribution of the larger radius 10% to the dissolution will be small except at the very end of the dissolution process and the particles at the small end of the distribution will dissolve very rapidly. Consequently, after they have dissolved completely, these smallest particles will contribute nothing to the solution of Eq. 9. Therefore, deviations from the log-normal case resulting from the use of Eq. 15 are modest, and the general conclusions from its use may be considered to also be approximately true for the log-normally distributed case.

The Dissolution Rate of Powder when $n = K/a_o^4$.—Substituting from Eq. 15 into Eq. 10 and integrating gives

$$Q = \frac{4K\pi\rho}{3M_o}\left(ln\frac{a_{lo}+a_l}{a_{ot}} - \frac{a_l}{a_{lo}} - \frac{a_l^3}{3a_{lo}^3}\right) \quad \text{(Eq. 16)}$$

where

$$a_l = \sqrt{a_{lo}^2 - \frac{2D\Delta Ct}{\rho}}$$

In our hypothetical case we do not have an experimental value for M_o so we must use Eq. 7, which gives

$$M_o = 4/3\pi\rho K\, ln\frac{a_{lo}}{a_{so}} \quad \text{(Eq. 17)}$$

and

$$Q = \frac{1}{ln\left(\frac{a_{lo}}{a_{so}}\right)}\left(ln\frac{a_{lo}+a_l}{a_{ot}} - \frac{a_l}{a_{lo}} - \frac{a_l^3}{3a_{lo}^3}\right) \quad \text{(Eq. 18)}$$

In a real case it might be better to evaluate K to give the best fit to the real size distribution and use the experimental value of M_o in the equation. Of course this leads to some hypothetical values of the largest and smallest radii that correspond with the selected value of K. These we may designate a_{lK} and a_{sK}, respectively. It is necessary that $a_{lK} \geqslant a_{lo}$ and that $a_{sK} \leqslant a_{so}$ where a_{lo} and s_{so} now define the experimental largest and smallest particle in the real sample.

An Illustrative Example.—Figure 2 represents a plot of Eq. 18 when the following values are used: $a_{lo} = 10\mu$; $a_{so} = 0.3\mu$; $\rho = 1.3$ Gm/ml.; $\Delta C = 1 \times 10^{-4}$ Gm./ml.; $D = 5 \times 10^{-6}$ cm²./sec. Note that Q *vs.* log t gives a nearly linear plot over much of the dissolution time.

According to Eq. 4, a_{so} will dissolve completely when

$$t = \frac{(3 \times 10^{-5})^2\, 1.3}{2 \times 5 \times 10^{-6} \times 1 \times 10^{-4}} = 1.17 \text{ sec.}$$

and a_{lo} will dissolve completely when

$$t = \frac{(10 \times 10^{-4})^2\, 1.3}{2 \times 5 \times 10^{-6} \times 1 \times 10^{-4}} = 1300 \text{ sec.} = 21 \text{ min. and } 40 \text{ sec.}$$

Also, Fig. 2 shows the dissolution rate of a material if the entire material is assumed to have the radius of the mass median radius. Obviously, the results of a calculation based on a single particle size do not produce rates similar to those that include the entire size range.

Figure 3 shows the change of a_l and a_{ol} during the dissolution process.

Figure 4 represents the size distributions at the time when approximately half of the solid has dissolved. Obviously, the smallest particles are dissolving most rapidly and the size distribution changes accordingly.

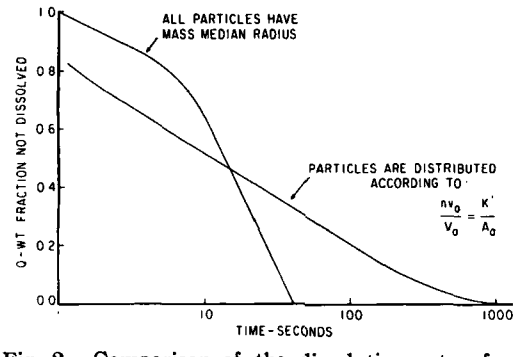

Fig. 2.—Comparison of the dissolution rate of a polydispersed powder with a monodispersed powder.

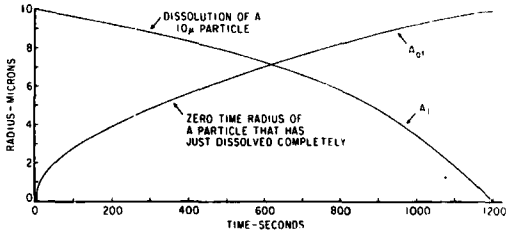

Fig. 3.—Changes in a_l and a_{ol} with time.

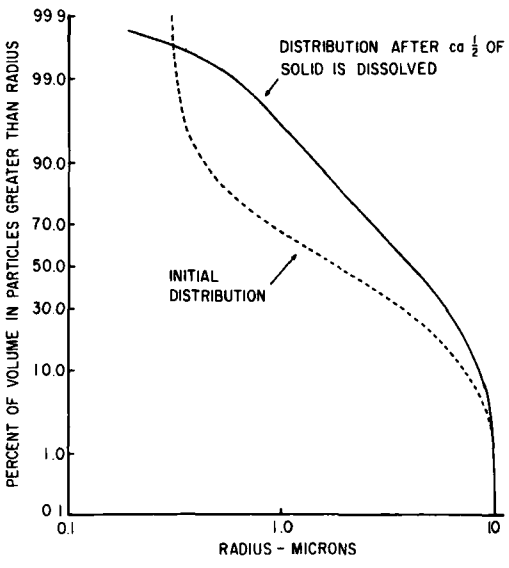

Fig. 4.—Illustration of size distribution changes during dissolution of powder.

Equations 13, 15, and 18 are combined to obtain Eq. 19.

$$> a = \frac{ln \frac{a_{lo} + a_l}{a_o + a} + \frac{a}{a_o} - \frac{a_l}{a_{lo}} + \frac{a^3}{3a_o^3} - \frac{a_l^3}{3a_{lo}^3}}{Q \, ln \frac{a_{lo}}{a_{so}}} \qquad \text{(Eq. 19)}$$

which was used to calculate this plot.

DISCUSSION

In the derivation of Eq. 18, ΔC was considered constant both with time and with particle size. The first requires that the liquid phase be large so that no significant change in concentration results from the dissolved solid. A later communication (1) describes a method of handling this problem when a correction is needed.

The particle size effect on solubility cannot be easily included since many sizes are present at any given time. If one assumes the interfacial tension to be about 50 ergs/cm.^{2}, then a 0.1 μ particle would have a solubility approximately 1.1 times that of a large particle. Usually this does not become significant except when dissolving in a nearly saturated vehicle. Such small particles dissolve so rapidly even when the change in ΔC is neglected that this effect cannot significantly alter the dissolution characteristics of the powder.

APPENDIX

Considerations on the Validity of Equation 2.— The use of the steady state equation to describe this case seems to be justified. The arguments used by Nielsen (2) for the case of crystal growth may be applied here since the growth and dissolution expressions are identical except for sign, as becomes evident in the following:

Fick's law of diffusion is

$$\frac{\partial C}{\partial t} = D\nabla^2 C \qquad \text{(Eq. 1a)}$$

When solved for the boundary conditions for growth it gives

$$C(r,t) = C_o \left[1 - \frac{a}{r} \, erfc \frac{r - a}{2\sqrt{Dt}}\right] \qquad \text{(Eq. 2a)}$$

where

$$erfc \, y = \frac{2}{\sqrt{\pi}} \int_y^\infty e^{-E^2} dE$$

and when solved for the boundary conditions for dissolution it gives

$$c(r,t) = C_o \left[\frac{a}{r} \, erfc \frac{r - a}{2\sqrt{Dt}}\right] \qquad \text{(Eq. 3a)}$$

and from Eq. 1 the gradient across the boundary sphere at $r = a$ is

$$\frac{dm}{dt} = 4\pi a^2 D \left(\frac{\partial C}{\partial r}\right)_{r=a}$$

Obviously, $\partial c/\partial r$ for the two cases differ only in sign.

Equation 2 may be written to include the time dependent term. It becomes

$$\frac{dm}{dt} = 4\pi D \Delta C a \left(1 + \frac{a}{\sqrt{\pi Dt}}\right) \qquad \text{(Eq. 4a)}$$

where the second term in the parenthesis represents the non-steady part, *i.e.*, the time dependent rate. If this term becomes much less than the first term before any significant material has dissolved then the steady state case is a very good approximation.

An estimate of the time, t', required for this term to be negligible is obtained when

$$\int_o^{t'} dt \gg \int_o^{t'} \frac{a dt}{\sqrt{\pi Dt}} \qquad \text{(Eq. 5a)}$$

which gives

$$t' \gg 2a \left(\frac{t'}{\pi D}\right)^{1/2}$$

or

$$t' \gg \frac{4a^2}{\pi D} \qquad \text{(Eq. 6a)}$$

When $a = 1 \times 10^{-4}$ cm. and $D = 5 \times 10^{-6}$ cm.^{2}/sec. as before, then

$$t' \gg 2 \times 10^{-3} \text{ sec.}$$

is the criterion of the time in which very little dissolution should occur if the steady state equation is to be sufficient.

Both Eqs. 4 and 6a involve a^2, so the time criterion changes with the radius in the inverse direction to the rate of change of radius by dissolution. Thus, the effects are compensating.

Equation 4a rigorously applies only when a is constant. Obviously a is constant only when $\Delta C = o$ and in a real case $\Delta C \neq o$. Therefore, one must determine how large ΔC may be before the change in a is important.

The steady state diffusion layer will have a thickness at least equal to a. A significant change in a might be set at level where the change in a is 1% or more of the diffusion layer thickness, *i.e.*, approximately 0.01 a. The change in a necessary to establish the diffusion layer may be estimated by estimating the amount of solid required to form the diffusion layer. Of course, this varies with the magnitude of ΔC. In the case considered here, values of $\Delta C < 1 \times 10^{-2}$ Gm./ml. seem to be sufficient for the constancy of a to be a good approximation for calculating t'.

DEFINITIONS OF SYMBOLS

a = radius of a particle at time t.
a_o = radius of a particle at zero time.
a_{lo} = radius of largest particle at zero time.
a_{so} = radius of smallest particle at zero time.
a_{ot} = zero time radius of largest particle that has completely dissolved at time t.
a_l = $\left(a_{lo}^2 - \frac{2D\Delta Ct}{\rho}\right)^{1/2}$
a_{lK} = hypothetical values of the largest particle corresponding to the value of K.
a_{sK} = hypothetical values of the smallest particle corresponding to the value of K.

a_i = any chosen value of a in the range present.
a_{io} = the zero time radius of a_i.
C = concentration.
C_s = saturation concentration.
C_o = concentration of bulk of solution.
D = diffusion coefficient.
$f>a_o$ = fraction larger than radius, a_o, at zero time.
$f>a$ = fraction larger than radius, a, at time t.
K = a proportionality constant.
$K' = \frac{4\pi K}{3V_o}$
m = mass of a single particle.
M = total mass of undissolved solid.
M_o = total mass of solid at zero time.
n = number of particles of a given size.
N = total number of particles.
Q = fraction of undissolved solid at time t; M/M_o.
r = radius of imaginary sphere through which diffusion occurs.
t = time.
v = volume of a single particle.
$V_o = \frac{M_o}{\rho}$
v_o = volume of a particle of a_o radius at zero time.
π = 3.1416.
ρ = density.

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(1) Higuchi, W. I., Rowe, E. L., and Hiestand, E. N. THIS JOURNAL, in press.
(2) Nielsen, A. E., *J. Phys. Chem.*, **65**, 46(1961).

Hypotensive Activity of Certain Diquaternarized Ammonium Compounds as Influenced by Administration Route and Anesthesia

By DAVID W. COATES†, JOSEPH P. BUCKLEY, and WILLIAM J. KINNARD

A three-way crossover experimental design demonstrated that urethan anesthesia enhanced the hypotensive responses of certain diquaternarized ammonium compounds in normotensive rats. The test showed that cardiovascular responses were more significant in hypertensive than in normotensive rats. Barbiturate anesthesia potentiated the hypotensive responses of certain of the compounds in normotensive dogs. Intravenous infusion of two of the compounds into anesthetized normotensive dogs produced a maximum hypotensive effect in 2 minutes with no further lowering of the blood pressure as the infusion was continued.

THE INCOMPLETE absorption following the oral administration of bisquaternary ammonium compounds has long been considered one of the main factors contributing to the erratic results produced by these ganglionic blocking agents in the treatment of arterial hypertension, and strong support for this view was provided by the absorption studies of Levine, *et al.* (1), and Schanker, *et al.* (2). Maxwell, *et al.* (3), reported the relative ineffectiveness of chlorisondamine in lowering the blood pressure of unanesthetized normotensive rats and dogs following intravenous administration. Haas and Goldblatt (4) obtained slight pressor responses in mean femoral arterial blood pressure following the intravenous infusion of tetraethylammonium, hexamethonium, pentolinium, chlorisondamine, and mecamylamine in unanesthetized normotensive dogs. However, these same investigators demonstrated a depressor response with pentolinium during an intermediate period of renal hypertension in dogs (5).

This report deals with the attempt to evaluate several bisquaternary ammonium compounds (Fig. 1) for their oral hypotensive activity in unanesthetized normotensive rats and dogs and in unanesthetized renal hypertensive rats. The experiment was designed to allow comparisons between oral and parenteral administration of the compounds to unanesthetized animals and between parenteral administration to unanesthetized and anesthetized animals.

METHOD

Hypotensive Activity in Normotensive Rats.—Normotensive Wistar rats were trained for indirect systolic blood pressure determinations using the photoelectric tensometer.[1] These rats were then divided into five groups of eight animals each. One of the following compounds was assigned to each

[1] Metro Industries.

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This investigation was supported in part by a research grant from Lakeside Laboratories, Milwaukee, Wis., and by Grant No. H-3475 from the National Heart Institute, Public Health Service, Bethesda, Md.
Presented to the Scientific Section, A.PH.A., Las Vegas meeting, March 1962.
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TABLE I.—EFFECTS OF CERTAIN HYPOTENSIVE AGENTS ON SYSTOLIC BLOOD PRESSURE OF NORMOTENSIVE RATS

Compound JB	Dose, mg./Kg.	Route of Adm.	Control Blood Pressure,[a] mm. Hg	% of Control Blood Pressure at Time Noted (min.) Following Drug Administration[a] 15	30	60	90	120	150	180	210	240
654	100	oral	118	114	111	110	117	111	116	125	114	119
654	10	i.p.	124	83	103	110	115	117	110	96	105	106
654	10	i.p.[b]	109	80	73	69	76	80	82	84	86	94
643	100	oral	108	91	104	98	98	92	103	98	93	99
643	10	i.p.	113	86	89	101	98	93	94	95	91	102
643	10	i.p.[b]	107	84	80	73	77	84	90	89	93	101
591	250	oral	104	96	97	92	91	91	106	104	97	100
591	25	i.p.	107	86	89	99	96	94	86	93	98	89
591	25	i.p.[b]	101	83	82	84	88	87	99	107	95	97
676	75	oral	124	91	93	92	89	89	92	96	99	99
676	7.5	i.p.	131	96	99	97	98	101	103	106	96	108
676	7.5	i.p.[b]	124	65	65	65	72	73	78	83	75	90
Chlorisondamine	5	oral	115	103	100	106	107	103	104	104	104	106
Chlorisondamine	0.5	i.p.	121	85	94	98	98	98	104	97	98	101
Chlorisondamine	0.5	i.p.[b]	108	71	59	61	64	70	77	87	90	95

[a] Each value in the table represents the mean responses of eight rats. [b] Rats anesthetized with urethan, 1.2 Gm./Kg. i.p.

group: JB 654, JB 643, JB 591, JB 676, or chlorisondamine dimethochloride.[2] The compounds were first administered orally to 24-hr. fasted unanesthetized animals in a dose ten times the selected intraperitoneal dose for that particular compound (6). The oral doses selected were as follows: JB 654, 100 mg./Kg.; JB 643, 100 mg./Kg.; JB 591, 250 mg./Kg.; JB 676, 75 mg./Kg.; and chlorisondamine dimethochloride, 5 mg./Kg. Blood pressures were obtained prior to administration of the compounds and periodically as indicated in Table I. After a recovery period of from 3 to 5 days to allow for full elimination of the compounds, these same unanesthetized groups of rats received the compounds by intraperitoneal injection. Blood pressure determinations were again made over the noted period of time. Following the recovery period, these same groups of rats were anesthetized with urethan, 1.2 Gm./Kg. i.p., and control pressures obtained. The compounds were administered by intraperitoneal injection and the effects on systolic blood pressure determined.

All compounds were administered in the form of freshly prepared solutions in distilled water. The concentrations were held constant for each compound, since it has been shown that the concentration is an important factor in oral absorption (7, 8).

The data were grouped into three sections: results following oral administration, results following intraperitoneal administration in unanesthetized rats, and results following intraperitoneal administration in anesthetized rats. The data were then subjected to an analysis of variance (9) to determine any differences between the actions of the compounds within each of the three sections.[3] The analysis also made it possible to demonstrate any effect time had upon the results obtained.

The data were then regrouped and paired comparisons (10) made between the oral and the intraperitoneal unanesthetized results and the intraperitoneal unanesthetized and intraperitoneal anesthetized results for each compound.[4] The values for the comparisons were obtained by totaling the blood pressure responses, noted as the per cent of the control blood pressure, at each time interval following drug administration and dividing by the number of intervals for each of the eight rats in the groups. This can be termed: the Individual Overall Cardiovascular Response (IOCR). A single figure representative of the entire group was determined by

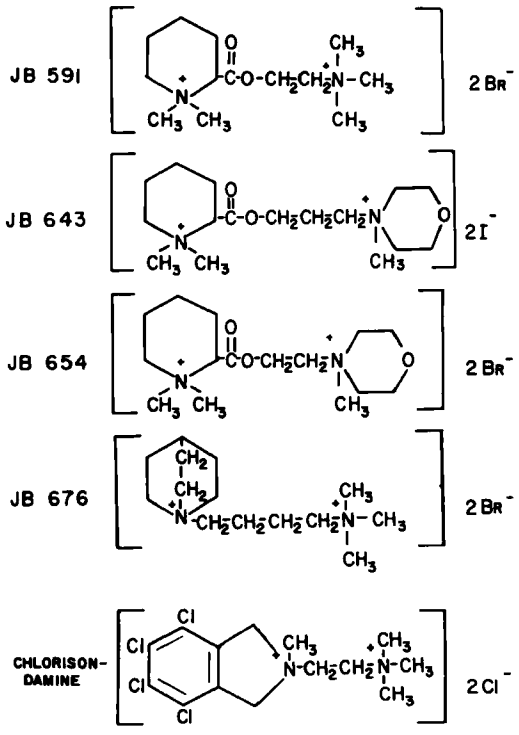

Fig. 1.—Structures of compounds investigated. JB 591 (β-dimethylaminoethyl N-methylpipecolinate dimethobromide), JB 643 (3-morpholinopropyl N-methylpipecolinate dimethoiodide), JB 654 (2-morpholinoethyl N-methylpipecolinate dimethobromide), JB 676 (N-(4-trimethylaminobutyl)-quinuclidinium bromide methobromide), and chlorisondamine (4,5,6,7-tetrachloro-2 (dimethylaminoethyl) isoindoline dimethochloride).

[2] Supplied as Ecolid by Ciba Pharmaceutical Products.
[3] Significance was measured at P = 1%.
[4] Significance was measured at P = 0.05.

TABLE II.—EFFECTS OF CERTAIN HYPOTENSIVE AGENTS ON SYSTOLIC BLOOD PRESSURE OF HYPERTENSIVE RATS

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654	10	i.p.[b]	137	40	40	45	44	53	64	67	73	79
643	100	oral	195	84	73	72	71	70	63	68	70	73
643	10	i.p.	185	60	64	71	83	89	92	94	93	94
643	10	i.p.[b]	140	48	45	60	63	76	82	83	86	90
591	250	oral	214	80	86	76	74	78	78	80	80	82
591	25	i.p.	211	51	67	78	73	83	90	80	91	92
591	25	i.p.[b]	181	62	53	60	64	71	73	78	81	82
676	75	oral	210	79	78	75	83	79	87	82	88	91
676	7.5	i.p.	204	89	107	97	108	105	108	108	106	108
676	7.5	i.p.[b]	171	58	61	73	80	82	84	92	95	95
Chlorisondamine	5	oral	200	101	104	104	108	116	117	115	117	115
Chlorisondamine	0.5	i.p.	214	103	83	99	106	106	110	107	107	107
Chlorisondamine	0.5	i.p.[b]	236	44	49	33	46	42	46	56	61	66

[a] Each value in the table represents the mean responses of eight rats. [b] Rats anesthetized with urethan 1.2 Gm./Kg. i.p.

dividing the sum total of the IOCR's by the number of subjects in the group. This was termed: Overall Cardiovascular Response (OCR) for that particular compound and method of administration. An OCR less than 100 indicated hypotensive activity while a figure greater than 100 indicated pressor activity.

Hypotensive Activity in Hypertensive Rats.—Hypertensive rats were prepared using a modification of the method of Freed, *et al.* (11). Four to 6 weeks following the simultaneous unilateral nephrectomy and figure-of-eight ligature of the contralateral kidney, weekly injections of desoxycorticosterone trimethylacetate,[5] 0.25 ml. (6.25 mg.), were initiated. Simultaneously, 1% sodium chloride solution was substituted for drinking water. This regimen was continued until a marked and sustained hypertension was produced, as recorded by the photoelectric tensometer. The rats were divided into five groups of eight animals each, and the identical experimental design as that outlined for normotensive rats was employed.

Hypotensive Activity in Normotensive Dogs.—The systolic blood pressures of unanesthetized mongrel dogs were obtained by the method of Prioli and Winbury (12) using the Infratron unit.[6] The dogs were restrained in a specially constructed animal sling which limited movement but did not completely support the animals. The tail was shaved and the coccygeal artery, located on the ventral surface, used as the site of blood pressure determination. A digital cuff was placed at the base of the tail and the microphone of the Infratron unit secured to the tail beneath the coccygeal artery, distal to the cuff, and so adjusted to obtain the best pulse pattern that could be observed on an oscilloscope. Systolic blood pressure was determined by increasing the cuff pressure until the pulse waves disappeared and then slowly reducing this pressure until the pulse pattern was again evident on the oscilloscope. At this point, the cuff pressure in mm. of Hg was recorded as the systolic blood pressure. The experimental design was identical with that utilized in rats except that the number of animals per group was reduced to four, 35 mg./Kg. i.v. of pentobarbital sodium was utilized as the anesthetic, and the compounds were administered orally in capsule form or intravenously.

Intravenous Infusion of JB 591 and JB 676.—Mongrel dogs were anesthetized with 35 mg./Kg. i.v. of pentobarbital sodium and prepared for direct femoral blood pressure determinations in the usual manner. The contralateral femoral vein was isolated and freed of surrounding fascia. An intravenous drip system[7] was fitted with a 20-gauge hypodermic needle and adjusted to deliver 1 ml./min. of the drug solutions. Aqueous drug solutions were prepared in suitable concentrations so that a previously determined total intravenous dose of JB 676 (7.5 mg./Kg.) or JB 591 (25 mg./Kg.) could be delivered over a 20-min. period. Following a stabilization period of at least 30 minutes, the needle was inserted into the vein and the infusion started. Blood pressure recordings were obtained for the 20-min. infusion period.

RESULTS

Hypotensive Activity in Normotensive Rats.—The effects of the diquaternary ammonium compounds on the blood pressure of normotensive rats are summarized in Table I. The compounds did not produce significant hypotensive effects when administered orally or intraperitoneally to unanesthetized normotensive rats. There was no significant difference between any of the compounds when the IOCR data for oral and intraperitoneal activity were analyzed.

Urethan anesthesia enhanced the hypertensive activity of JB 654, JB 676, and chlorisondamine but did not significantly alter the depressor response to JB 591 and JB 643.

Hypotensive Activity in Hypertensive Rats.—The results obtained following the administration of the compounds to hypertensive rats are summarized in Table II. Most of the compounds were active in unanesthetized hypertensive rats in contrast to their relative inertness in unanesthetized normotensive rats. The analysis of variance demonstrated a difference between the activities of the compounds

[5] Supplied as Percorten by Ciba Pharmaceutical Products.
[6] Medical Electronics Development Co.
[7] Marketed as Venopak by Abbott Laboratories.

when administered orally. The OCR's, expressed in per cent of predrug control blood pressure, suggested the following order of decreasing potency: JB 643, 72%; JB 591, 79%; JB 676, 82%; JB 654, 88%; and chlorisondamine, 112%. The maximum hypotensive effects occurred in approximately 60 minutes with JB 591 and JB 676 and 150 minutes with JB 643. There was a marked difference in the response to certain of the compounds when they were administered intraperitoneally to unanesthetized hypertensive rats. The activities of JB 654, JB 643, and JB 591 were similar, their respective OCR's being 82%, 82%, and 79% of the control blood pressure. JB 676 and chlorisondamine produced slight pressor responses. The maximum mean hypotensive effects of the depressor compounds occurred 15 minutes following drug administration.

Analysis of the data obtained from the intraperitoneal administration of the compounds to anesthetized hypertensive rats showed a difference in the hypotensive activity of the compounds. All the agents were active and the following order of decreasing hypotensive activity was suggested: chlorisondamine and JB 654, 50% and 56% of the control; JB 591 and JB 643, 69% and 72%; and JB 676, 80%. Maximum hypotensive activity occurred within 15 minutes for all the compounds except JB 591, which required 30 minutes.

The results of the paired comparisons between the oral and intraperitoneal routes of administration in unanesthetized animals showed the oral route to be significantly more effective in lowering the blood pressure for JB 643 and JB 676. The intraperitoneal route was more active for JB 654 and chlorisondamine, and there was no significant difference between these two routes of administration with JB 591.

When the data obtained from the intraperitoneal administration of the drugs to unanesthetized and anesthetized rats were compared, urethan anesthesia was found to significantly enhance the hypotensive activity of all compounds in the hypertensive animals.

Hypotensive Activity in Normotensive Dogs.—All the compounds produced some degree of hypotension in normotensive dogs (Table III). However, the differences in activity were not significant regardless of the route of administration. The OCR's, reported in per cent of predrug blood pressure, following oral administration were: JB 654, 75%; JB 643, 83%; JB 591, 89%; JB 676, 88%; and chlorisondamine, 94%. The mean depressor responses were maximal at the termination of the experiment (240 minutes).

The intravenous administration of the compounds to unanesthetized dogs produced the following OCR's: JB 654, 79% of the control pressure; JB 643, 81% of the control; JB 591, 75%. The time at which the maximum mean hypotensive effects occurred varied from 15 minutes for JB 654 to 180 minutes for JB 643. The intravenous administration of the compounds to anesthetized normotensive dogs produced the following OCR's: JB 654, 64%; JB 591, 70%; JB 643, 74%; chlorisondamine, 81%; and JB 676, 84%. Time of maximum depressor activity varied from 15 minutes for JB 654 to 120 minutes for JB 643 and chlorisondamine.

The paired comparison of the data obtained with the compounds administered orally and intravenously to the unanesthetized dogs suggest that JB 676 and chlorisondamine were more effective intravenously and JB 654 more effective orally. No significant differences could be demonstrated between the intravenous or oral activities of JB 591 and JB 643.

Analysis of the data obtained when the compounds were administered intravenously to unanesthetized and anesthetized dogs indicated that the anesthetic augmented the hypotensive effects produced by JB 654, JB 591, and JB 676. The anesthetic did not significantly alter the depressor effect of JB 643 and chlorisondamine.

Intravenous Infusion of JB 591 and JB 676.—The results obtained from the intravenous infusion of JB 676 and JB 591 into anesthetized normotensive dogs are recorded in Table IV. JB 676 produced its maximum effect upon blood pressure within 2 minutes in each of the two dogs receiving this drug. The dose delivered at this time was one-tenth of the

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643	100	oral	134	92	87	83	81	78	76
643	10	i.v.	148	83	82	79	81	79	83
643	10	i.v.[b]	130	72	72	72	71	74	72
591	250	oral	155	88	103	83	84	89	82
591	25	i.v.	143	95	92	87	88	88	87
591	25	i.v.[b]	145	72	65	61	68	68	75
676	75	oral	125	92	88	83	94	87	83
676	7.5	i.v.	122	78	75	78	77	84	87
676	7.5	i.v.[b]	142	78	79	84	88	90	83
Chlorisondamine	5	oral	131	94	100	95	90	92	91
Chlorisondamine	0.5	i.v.	139	83	71	75	75	78	76
Chlorisondamine	0.5	i.v.	128	85	78	75	76	89	79

[a] Each value in the table represents the mean responses of four dogs. [b] Dogs anesthetized with pentobarbital sodium, 35 mg./Kg. i.v.

TABLE IV.—EFFECT OF AN INTRAVENOUS INFUSION OF JB 676 AND JB 591 ON BLOOD PRESSURE OF ANESTHETIZED NORMOTENSIVE DOGS

Compound JB	Control Blood Pressure, mm. Hg	% Drop in Blood Pressure at Time (min.) Following Start of Infusion 2	10	20[a]
676[b]	110	40	31	38
676[b]	180	20	19	19
591[c]	160	25	12	16
591[c]	154	31	30	30

[a] End of infusion period. [b] Infusion rate of 1 ml./min. (0.375 mg./Kg./min.); total dose, 7.5 mg./Kg. in 20 minutes. [c] Infusion rate of 1 ml./min. (1.25 mg./Kg./min.); total dose, 25 mg./Kg. in 20 minutes.

total dose or 0.75 mg./Kg. No further decrease in blood pressure was produced over the 20-minute infusion period.

JB 591 produced similar effects in two dogs. The maximum depressor response occurred in less than 2 minutes. The dose delivered at this time was 2.5 mg./Kg. No further decrease in the blood pressure was realized over the remainder of the infusion period. Total dose given was 25 mg./Kg.

DISCUSSION

The results obtained from the administration of the experimental compounds to unanesthetized normotensive rats and dogs indicate that there is some mechanism present in the normotensive animal which is capable of offsetting the expected hypotensive effects of ganglionic blocking agents. Since these agents are all active in anesthetized animals, the nature of the compensatory mechanism would appear to be central in origin. Most of the compounds were active in unanesthetized hypertensive rats indicating that these restorative mechanisms were reduced in the hypertensive animals but not absent since the compounds were even more active in anesthetized hypertensive rats.

No explanation can be offered for the ineffectiveness of chlorisondamine in unanesthetized hypertensive rats. This finding is similar to the results obtained by Maxwell, *et al.* (3), in studies on unanesthetized normotensive rats. However, when chlorisondamine was administered to these animals under barbiturate anesthesia, the compound produced marked sustained falls in both systolic and diastolic blood pressure. Haas and Goldblatt also studied the effects of various ganglionic blocking agents in normotensive (4) and hypertensive (5) dogs. Infusion of tetramethylammonium, hexamethonium, pentolinium, chlorisondamine, and mecamylamine in unanesthetized normotensive dogs resulted in slight pressor responses in mean femoral blood pressure. Pentolinium infusion produced varying effects in renal hypertensive dogs. They noted that pressor responses were obtained following short (2 to 14 days) and long (50 to 103 weeks) periods of hypertension but a depressor response was obtained during an intermediate period (2 to 19 weeks) of hypertension. It is possible that this same pattern of response to ganglionic blocking agents occurs in hypertensive rats. The hypertensive rats used in this study were prepared at approximately the same time and were possibly tested during this intermediate period. An explanation for the hypotensive activity of the experimental compounds and the ineffectiveness of chlorisondamine may be the possible central hypotensive actions of the former compounds. Although JB 654, JB 643, and JB 676 have not been tested in the cross circulation preparation, they are structurally related to JB 591 (see Fig. 1), which has been reported to produce a centrally evoked depressor response (13).

The results obtained from the intravenous infusion of JB 591 and JB 676 in anesthetized normotensive dogs demonstrated a difficulty which might arise in attempting to estimate oral absorption on the basis of activity produced. One-tenth of the previously employed intravenous doses of these compounds produced a maximum hypotensive effect on blood pressure. The remainder of the dose must, therefore, contribute only to maintaining the duration of this maximum effect. If this is also true of oral absorption, then we cannot estimate oral absorption in excess of the amount necessary to produce maximum effects.

SUMMARY

1. The three-way crossover design in normotensive rats demonstrated no significant hypotensive activity following either oral or intraperitoneal administration of the drugs to unanesthetized animals. Urethan anesthesia enhanced the hypotensive responses of JB 654, JB 676, and chlorisondamine.

2. There was a statistically significant difference between the hypotensive activities of the experimental compounds investigated in a three-way crossover test in hypertensive rats. The order of decreasing hypotensive activity following oral administration was: JB 643, JB 591, JB 676, and JB 654. Chlorisondamine produced a pressor response. JB 654, JB 643, and JB 591 produced approximately equal depressor effects upon intraperitoneal administration to unanesthetized hypertensive rats, JB 676 and chlorisondamine produced pressor effects, JB 643 and JB 676 were more active orally, and JB 654 more effective *via* the intraperitoneal route. JB 591 appeared equally active by either route. Urethan anesthesia increased the hypotensive response of all compounds.

3. All the compounds lowered systolic blood pressure of the normotensive dog regardless of the method of administration. The oral route was more active for JB 654 and the intravenous route was more active for JB 676 and chlorisondamine in unanesthetized dogs. Barbiturate anesthesia enhanced the hypotensive responses of JB 654, JB 591, and JB 676 but not those of JB 643 and chlorisondamine.

4. The intravenous infusion of JB 591 and JB 676 in anesthetized normotensive dogs showed the

maximum hypotensive response to occur within 2 minutes and at a dose one-tenth that previously employed. Continued infusion only served to maintain the hypotensive effect.

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Pharmaceutical Aspects of a *p*-Aminosalicylate Dialdehyde Starch Compound

By JAMES A. CAMPBELL

A compound resulting from the reaction between sodium *p*-aminosalicylate and dialdehyde starch was investigated. The bland taste and slow rate of dissolution of the compound appeared to offer certain advantages in the administration of NaPAS. The compound was subjected to certain *in vitro* and *in vivo* tests to demonstrate the availability of NaPAS.

THE EFFECT of *p*-aminosalicylic acid upon the tubercule bacillus has been known for a number of years. Bernheim (1) in 1941 observed that benzoic and salicylic acids increased the oxygen consumption of the bacillus. Lehmann (2) discovered that the increased oxygen consumption was accompanied by an inhibition in growth and multiplication, and subsequently found that *p*-aminosalicylic acid was the most effective of a group of related compounds.

A number of salts and other derivatives of the acid have been prepared and investigated (3, 4). Particularly well known are the sodium, potassium, and calcium salts. These salts have the advantage of being more soluble than the acid, and are reputed to be less irritating. Aqueous solutions of the sodium salt are more stable on heating than is a solution of the acid.

Foye and Duvall (5) made a comparative study of the *in vivo* antitubercular activity in mice of cupric and ferrous chelates of PAS. Both compounds were found to be active, but the cupric complex possessed a much higher activity than the ferrous complex.

Due to the large dosage level of PAS (2–4 Gm., with a daily average dose of 12–16 Gm.), the unpleasant taste, the irritant action, and other factors, the administration of PAS is sometimes beset with problems. Intolerance manifested by nausea and vomiting is common with average or large doses (6).

The objective in this effort was to prepare and study a derivative of PAS which might have certain advantages in overcoming the problems associated with the administration of PAS. Dialdehyde starch is known to react with amines and compounds containing the amino group, and the resulting compounds are sometimes noted for their unique properties (7). Dialdehyde starch was thus reacted with sodium PAS and the resulting compound was studied.

EXPERIMENTAL

Formation of the Compound.—A 27.4-Gm. quantity (0.141 mole) of dialdehyde starch (90.4% oxidized; 8.6% moisture) and 24.6 Gm. (0.116 mole) of sodium *p*-aminosalicylate dihydrate was slurried in 100 ml. of dry benzene in a 500-ml. three-necked flask equipped with a stirrer, Barret trap, and condenser. During the refluxing and stirring period of 5 hours, 5.0 ml. of water separated in the Barret trap. After cooling the mixture to room temperature, the pale yellow solid was collected, washed with dry benzene and dried to constant weight in a vacuum oven. Subsequent analysis including infrared spectra showed that a linkage between the compounds had taken place. Total nitrogen and free aldehyde content showed that NaPAS had added to half of the available aldehyde groups. Based upon the action of dialdehyde

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EXPERIMENTAL

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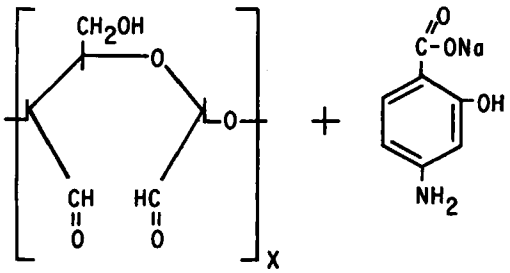
starch with other compounds similar to NaPAS (7), the linkage is believed to have occurred according to the equation shown in Fig. 1.

Description of the Compound.—The compound is a pale yellow to light tan powder, having a bland taste. One gram is soluble in 2.85 Gm. of water.



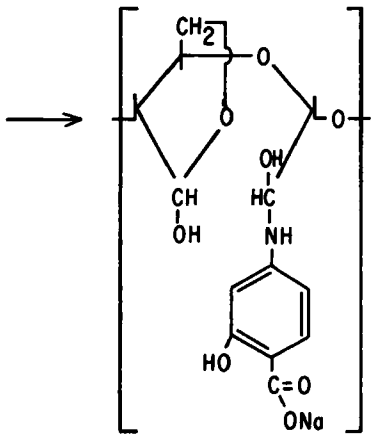


Fig. 1.—Reaction between dialdehyde starch and sodium *p*-aminosalicylate.

Prolonged-Release Experiments.—Accurately weighed, approximately 0.5 Gm., samples of the compound were sealed in small packets made of filter paper (Whatman #41). The packets were heat sealed after application of a heat sensitive adhesive. A packet was then placed in a 1-L. round-bottom flask containing 500 ml. of simulated gastric fluid, T.S. The fluid was stirred constantly with a mechanical stirrer which passed through the neck of the flask by means of a mercury seal. The flask was mounted in a water bath maintained at 37°. At the end of 1 hour and 20 minutes, half of the fluid was removed and replaced by an equal quantity of simulated intestinal fluid, T.S. At the end of 2 hours and 40 minutes, all the fluid was replaced by 500 ml. of fresh intestinal fluid, T.S. Small samples of the fluid were removed by means of a pipet at varying time intervals up to the end

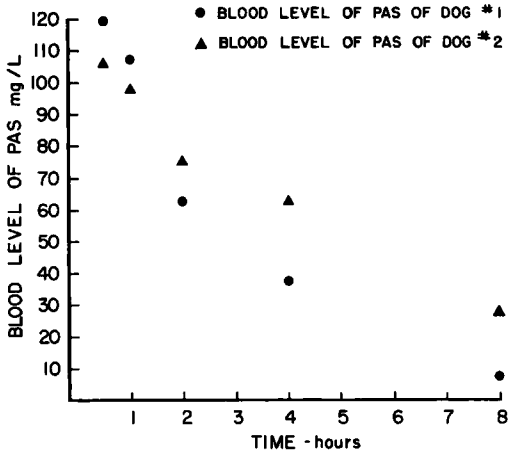


Fig. 3.—Blood levels of free PAS in dogs after oral administration of 100 mg./Kg. of free NaPAS.

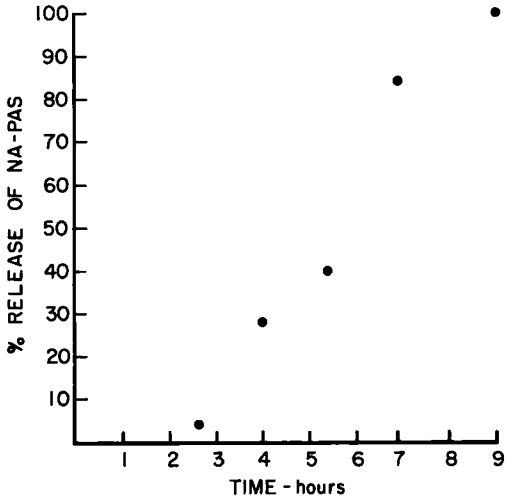


Fig. 2.—Rate of passage of NaPAS from a compound through a filter paper barrier when bathed in simulated gastrointestinal fluids.

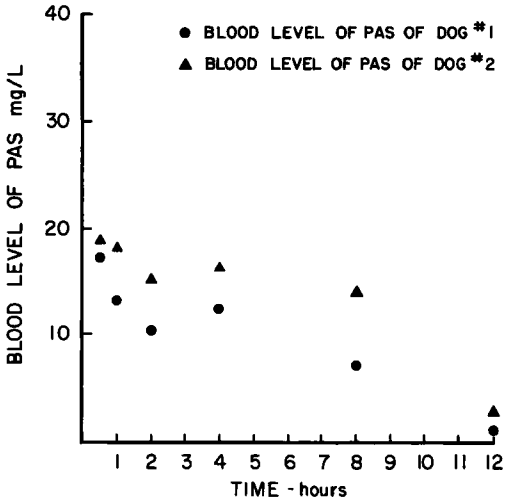


Fig. 4.—Blood levels of free PAS in dogs after oral administration of a quantity of a compound equivalent to 100 mg./Kg. of free NaPAS.

of a 9-hour period from the beginning of the experiment. Each time a sample was removed, an equal volume of fluid was placed back into the flask to maintain a constant volume.

The samples of fluid removed were used to assay for NaPAS content in the following manner. A 5-ml. quantity of the solution to be assayed was placed in a 250-ml. volumetric flask containing 12.5 ml. of concentrated pH 7 buffer (prepared by dissolving 34 Gm. of C.P. anhydrous KH_2PO_4 in 136 ml. of 1 *N* NaOH and diluting to 1000 ml. with distilled water). This was diluted to volume with distilled water and mixed well. A blank was prepared by diluting 5 ml. of concentrated pH 7 buffer to 100 ml. with distilled water. The absorbance of the sample solution was determined at 265 and 299 mμ in a 1.0-cm. quartz cell by means of a Beckman DU spectrophotometer using the blank solution (diluted pH 7 buffer) as the reference liquid.

Calculations

$X = A\text{-}265\ (136.1) = \%$ NaPAS based on absorbance at 265 mμ

$Y = A\text{-}299\ (208.8) = \%$ NaPAS based on absorbance at 299 mμ

$\frac{X + Y}{2} = \%\ C_7H_6NNaO_3$ on anhydrous basis

The rate of passage of NaPAS is shown in Fig. 2. When the free NaPAS was treated under identical conditions, all had passed out of the packet within 1 hour.

PAS Blood Level in Dogs.—Two dogs were administered NaPAS by intubation at a dosage level of 100 mg./Kg. Blood samples were taken at 0, 1/2, 1, 2, 4, and 8 hours. The free PAS content in whole blood was determined by a modified Bratton-Marshall method (8). The concentration of PAS found in the blood is given in Fig. 3. Approximately 10 days later, the compound was administered to the same dogs and with the dosage level being the same in regard to NaPAS content. Blood samples were taken on the same schedule as in the former experiment with additional samples being taken at 12 and 24 hours. The PAS blood levels found are shown in Fig. 4.

SUMMARY AND CONCLUSIONS

Sodium *p*-aminosalicylate was reacted with dialdehyde starch and the resulting compound was characterized. The compound was tested for the rate of passage of NaPAS through a filter paper barrier when bathed in gastrointestinal fluids ranging from all gastric fluid to all intestinal fluid. It was found that very little passed through in the presence of all gastric fluid, but the rate of passage began to increase as part of the fluid was changed to intestinal fluid. The rate of passage appeared to be proportional to the percentage of intestinal fluid. All was found to have passed through at the end of a 9-hour test period.

When the compound was administered to dogs, PAS was detected in the blood during a 12-hour period, but at a lower level than when the uncombined NaPAS was administered.

The bland taste of the compound provides a definite advantage in the problems associated with the administration of NaPAS; however, the lower rate of absorption in the bloodstream of the dog might possibly be considered a disadvantage. The question as to the efficacy of the compound in the *in vivo* control of tuberculosis is yet to be determined.

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Synthesis and Local Anesthetic Activity of Several Dialkylaminoalkyl Esters of Indole Carboxylic Acids

By SAMUEL ELKIN† and FRANCIS M. MILLER

The largest number of compounds synthesized for local anesthetic purposes are the alkamine esters of aromatic acids. In this work, two indole carboxylic acids were incorporated as the acid portion of the ester. All the compounds were effective as topical anesthetics, but had no action on unbroken skin. Nitration of indole-3-carboxylic acid reduced the duration of activity of the esters. The esters of indole-2-carboxylic acid showed a marked decrease in duration of local anesthetic activity.

At present, most of the compounds synthesized for local anesthetic purposes are alkamine esters of aromatic acids. They furnish the preponderant number of marketable local anesthetics.

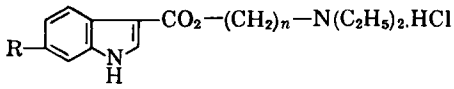
While procaine is relatively low in toxicity and very effective when given hypodermically (1), it requires the addition of a vasopressor substance to help localize the drug at the nerve and thereby prolong the period of anesthesia. Procaine is also only very slightly active as a topical anesthetic. Hence, many compounds of the same general type have been synthesized to improve these properties.

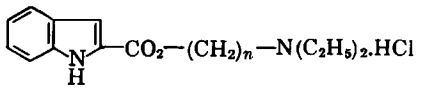
Among the modifications studied are included those which contain a heterocyclic aromatic ring in the acid portion of the molecule. Blicke and Jenner (2) prepared several dialkylaminoalkyl nicotinates, 2-alkyl acid quinolates, and 2-alkyl-3-dialkylaminoalkyl quinolates. They reported that none of these compounds possessed marked activity as local anesthetics.

Blicke and Blake (3) found that the local anesthetic action of a procaine-type compound can be retained by the substitution of a 2-pyrrolyl moiety for the benzoyl and 4-aminobenzoyl groups and by the replacement of dimethyl and diethylamino groupings by 1-pyrrolyl and 1-pyrollidinyl nuclei.

Burtner and Lehmann (4) investigated a series of alkylaminoalkyl esters of carbazole, dibenzfuran, and dibenzthiophene carboxylic acids. They prepared several effective local anesthetics. The best one of the series was β - diethylaminoethyl - 5 - ethylcarbazole - 3 - carboxylate hydrochloride. However, all of the foregoing were irritating and could not be regarded as useful anesthetics. An interesting conclusion which Burtner and Lehmann were able to derive from their investigations was that the activity of the compounds they prepared appeared to be predominantly a function of the carboxyl group rather than of other structural features. That is, equal concentrations of derivatives of carbazole-2- and carbazole-3-carboxylic acids were equally effective. However, the derivatives of carbazole-4-carboxylic acid showed no anesthetic activity.

In this investigation the following dialkylaminoalkyl esters of indole-3-carboxylic acid and indole-2-carboxylic acid have been prepared





I, R = H, n = 2; II, R = H, n = 3; III, R = NO_2, n = 2; IV, R = NO_2, n = 3; V, n = 2; VI, n = 3. The effectiveness of these compounds as local anesthetics has been determined. II showed the most pronounced activity followed by I.

The esters were prepared essentially according to the method of Burtner and Cusic (5). The indole carboxylic acids were dissolved in hot isopropanol and either 2-diethylaminoethyl chloride or 3-diethylamino-1-propyl chloride was added to the refluxing solution. The mixture was allowed to reflux for $2^1/_2$ hours and then cooled overnight. The method of isolation of the resulting compounds varied because of the difference in their solubility.

EXPERIMENTAL

Indole-3-carboxylic Acid (6, 7).—Into a thoroughly dried 1-L. flask equipped with stirrer, dropping funnel, and condenser (drying tube) were placed 12 Gm. (0.5 mole) of magnesium turnings, 100 ml. of anhydrous ether, and a small crystal of iodine as catalyst. Ethyl iodide, 5 Gm., was added all at

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TABLE I.—COMPILED DATA ON THE ESTERS PREPARED

Ester	Yield, %	M.p., °C.	Formula	Analysis N Calcd.	Analysis N Found
I	25.0	174–175	$C_{15}H_{21}N_2O_2Cl$	9.44	9.27
II	21.8	165–167	$C_{16}H_{23}N_2O_2Cl$	9.01	8.80
III	15.6	210–212	$C_{15}H_{20}N_3O_4Cl$	12.30	12.62
IV	15.1	208–210	$C_{16}H_{22}N_3O_4Cl$	11.81	12.13
V	6.2	178–180	$C_{15}H_{21}N_2O_2Cl$	9.44	9.31
VI	17.5	170–171	$C_{16}H_{23}N_2O_2Cl$	9.01	9.06

once and, after a few moments, the yellow color of the iodine disappeared and the ether began to boil. The reaction was controlled by periodic cooling in an ice bath. An additional 75 Gm. (0.5 mole, total) of ethyl iodide was then added dropwise. Stirring was maintained after the addition of the ethyl iodide for about 1/2 hour or until the reaction mixture cooled to room temperature.

An ice-salt bath was applied and 29.5 Gm. (0.25 mole) of indole in 40 ml. of anhydrous ether was added gradually with vigorous stirring. Ethyl chlorocarbonate, 30 Gm. (0.28 mole), was then added dropwise to the cooled solution of indolylmagnesium iodide. The mixture was kept cold and stirring was maintained for an hour after the addition of the ethyl chlorocarbonate. Ice water was added very slowly and the resulting mixture was acidified with acetic acid and the ether layer separated. The ether solution was washed with sodium bicarbonate and dried over sodium sulfate.

Removal of the ether under reduced pressure left a red oily residue of the ethyl ester. This was not purified, but was hydrolyzed by refluxing for 2 1/2 hours with 100 ml. of 0.5 *N* potassium hydroxide. The aqueous solution was separated from a gummy residue that formed, acidified with acetic acid whereby indole-3-carboxylic acid precipitated. The product was transferred to a beaker and washed with a little ether and petroleum ether to remove any unreacted indole. The acid was filtered and recrystallized from a 40% aqueous ethanol solution. Yield, 19 Gm. (47.5%); m.p., 218–220°. Reported m.p., 218–220° (6); 220–224° (8).

6-Nitroindole-3-carboxylic Acid.—Ten grams (0.06 mole) of indole-3-carboxylic acid was suspended in 50 ml. of glacial acetic acid contained in a 600-ml. beaker. A 50-ml. quantity of concentrated nitric acid was added dropwise with stirring. During the addition of the nitric acid the mixture turned a deep red and began to boil. The reddish-brown residue was collected after cooling overnight. This was dissolved in hot ethanol and treated with charcoal. After filtering, water was added to the alcoholic solution and a bright yellow product precipitated. Yield, 4 Gm. (31.2%); darkened at 223° and decomposed at 275–280°. Reported, darkening at 227° and decomposing at 275–280° (7).

Indole-2-carboxylic Acid.—Prepared from *o*-nitrophenyl-pyruvic acid (9) upon boiling with ferrous sulfate in ammonium hydroxide (10). M.p. 197–200°. Reported m.p., 202–204° (10).

2-Diethylaminoethyl Chloride.—Prepared according to Shirley (11) from 2-diethylaminoethyl chloride hydrochloride by treatment with sodium hydroxide and extraction with ether; b.p., 146–148°/756 mm. Reported b.p., 146–147°/750 mm. (11).

3-Diethylamino-1-propyl Chloride.—Prepared in the same manner as indicated under 2-diethylaminoethyl chloride. The free base, a colorless oil distilled at 94–95°/42 mm. Reported b.p., 82°/28 mm. (12).

2-Diethylaminoethyl Indole-3-carboxylate Hydrochloride (I)[1].—Into a 300-ml. flask equipped with stirrer, dropping funnel, and condenser were placed 5 Gm. (0.031 mole) of indole-3-carboxylic acid and 80 ml. of isopropanol. The acid dissolved upon heating. The solution was allowed to reflux and 4.2 Gm. (0.03 mole) of 2-diethylaminoethyl chloride was added dropwise. The mixture was refluxed for 2 1/2 hours and allowed to cool overnight. A white precipitate formed which was filtered off and washed with anhydrous ether. Recrystallization from isopropanol gave a product which was very readily soluble in water.

3-Diethylaminopropyl Indole-3-carboxylate Hydrochloride (II).—Into a 300-ml. flask equipped with stirrer, dropping funnel, and condenser were placed 4.5 Gm. (0.028 mole) of indole-3-carboxylic acid and 75 ml. of isopropanol. The acid dissolved upon heating. The solution was allowed to reflux and 4.2 Gm. (0.028 mole) of 3-diethylamino-1-propyl chloride was added dropwise. The mixture was refluxed for 2 1/2 hours and allowed to cool overnight. No precipitation resulted. The solvent was removed under reduced pressure leaving a reddish oil. The oil was washed several times with anhydrous ether whereupon it became semisolid and almost white. The compound was then treated with isopropanol and a small amount of anhydrous ether, and a white solid precipitated upon scratching. The product was recrystallized from absolute ethanol and ether.

All esters were prepared in a manner described above. Pertinent data are presented in Table I.

Pharmacological Procedures.—The hair around the rabbit's eye was carefully clipped. The winking reflex was tested by gently touching the center of the cornea with a bluntly pointed glass rod. Four drops (equivalent to 1.4 mg.) of 1% aqueous test

TABLE II.—LOCAL ANESTHETIC ACTIVITY OF THE DIALKYLAMINOALKYL ESTERS PREPARED

Ester	Trials	Minimum Duration, min.	Maximum Duration, min.	Average
I	35	68	152	94.2
II	18	83	141	117.0
III	6	49	73	57.4
IV	11	64	105	80.7
V	8	5	11	7.0
VI	7	25	47	36.0

[1] *Note added in galley proof:* J. Büche, *et al.*, *Arch. Pharmazie*, **295**, 209(1962), report the preparation of 2-diethylaminoethyl indole-3-carboxylate hydrochloride and the corresponding 6-nitro derivative.

solution was instilled into the conjunctival sac of the left eye by means of a pipet (28 drops per ml.). The right eye was maintained as a control. The solution was allowed to remain in contact with the surface of the eyeball for 2 minutes by gently pressing the lids together. The time of abolishment of the wink reflex was noted and the duration of anesthesia was tested at 2-minute intervals.

RESULTS

None of the compounds gave any evidence of any deleterious action on the eye. The eyes were carefully checked for corneal pitting, excessive lacrimation, and hyperemia. None of these was present. Pharmacological data are presented in Table II.

SUMMARY

Several dialkylaminoalkyl esters of two indole carboxylic acids were prepared. All of the compounds were effective as topical anesthetics, but had no action on unbroken skin. Nitration of indole-3-carboxylic acid reduced the duration of activity of the esters. The esters of indole-2-carboxylic acid showed a marked decrease in duration of local anesthetic activity.

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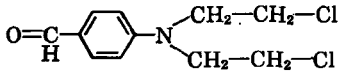

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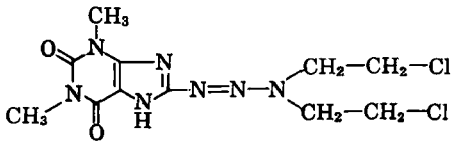

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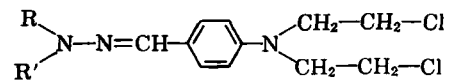

III

The preparation of III, together with the starting materials used, is described in the experimental section.

EXPERIMENTAL[1]

Synthesis of Hydrazinopyrimidines.—To a mixture of 35 Gm. of chloropyrimidine in 70 ml. of absolute ethanol was added, with stirring, 35 ml. of anhydrous hydrazine at such a rate that the reaction temperature remained at 65–75° (occasional cooling in an ice bath may be necessary). After the addition was complete, the resulting solution was stirred at room temperature for 30 minutes, during

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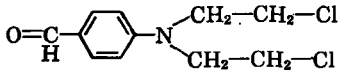

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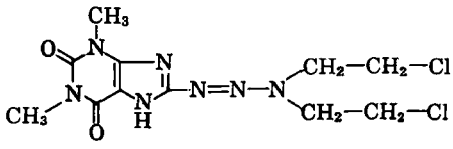

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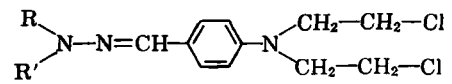

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[1] All melting points were taken on the Thomas-Hoover melting point apparatus.

which time crystallization took place. The reaction mixture was diluted with 200 ml. of ethanol and filtered. The solid product was washed well with ethanol and ether. The resulting hydrazinopyrimidine was of sufficient purity to be used for the preparation of hydrazones.

5-Bromo-4,6-di(hydrazino)pyrimidine was thus obtained from 5-bromo-4,6-dichloropyrimidine (9) in 46% yield, m.p. 150° dec.

Anal.—Calcd. for $C_4H_7BrN_6$: N, 38.3 Found: N, 38.5.

Similarly 5 - bromo - 4 - hydrazino - 2 - (methylthio) pyrimidine was prepared from 5-bromo-4-chloro-2-methylthiopyrimidine (10) in 35% yield, m.p. 148–150°.

Anal.—Calcd. for $C_5H_7BrN_4S$: N, 24.0. Found: N, 24.1.

2 - (Hydrazinomethyl)naphthimidazole.—To 75 ml. of anhydrous hydrazine was added, portionwise, 30 Gm. of 2-chloromethylnaphthimidazole [prepared by the method of Bloom and Day (11), using 2,3-naphthalenediamine rather than *o*-phenylenediamine] at such a rate that the reaction temperature remained below 80°. Crystallization began after the addition was complete. The reaction mixture was allowed to stand at room temperature for 20 minutes, diluted with 200 ml. of ethanol and filtered. The product was washed with ethanol and ether, and recrystallized from a mixture of dimethylformamide and water to give 14 Gm. of light yellow crystals, m.p. 245° dec.

Anal.—Calcd. for $C_{12}H_{12}N_4 \cdot H_2O$: N, 24.3. Found: N, 24.0.

5-Uracilcarbohydrazide was similarly prepared from 60 ml. of anhydrous hydrazine and 35 Gm. of 5-carbethoxyuracil (12) to yield, after recrystallization from dilute hydrochloric acid, 13 Gm. of white prisms, m.p. 245° dec.

Anal.—Calcd. for $C_5H_6N_4O_3 \cdot HCl$: N, 27.2. Found: N, 27.0.

General Method for the Preparation of *p*-[N,N-Bis(β - chloroethyl)amino]benzalhydrazone Derivatives (III).—To 250 ml. of absolute ethanol was added 0.04 mole of substituted hydrazine. The mixture was heated till solution took place (for those hydrazines that are insoluble in absolute ethanol, 80% aqueous ethanol was substituted). The solution was treated with charcoal and filtered. To the filtrate, heated at 60°, 0.04 mole of *p*-[N,N-bis(β - chloroethyl)amino]benzaldehyde (1, 2) dissolved in 250 ml. of absolute ethanol was added with stirring followed by two drops of concentrated hydrochloric acid. A precipitate appeared almost immediately. Stirring was continued for 5 minutes and the reaction mixture was filtered. The solid was washed with ethanol and ether, and dried *in vacuo* to give the desired product of analytical purity.

The following compounds were prepared:

2 - {*p* - [N,N - Bis(β - chloroethyl)amino]benzalhydrazono}pyrimidine, m.p. 187–188°, was prepared from 2-(hydrazino)pyrimidine (13) in 52% yield.

Anal.—Calcd. for $C_{15}H_{17}Cl_2N_5$: C, 53.2; H, 5.2; N, 20.7. Found: C, 53.5; H, 5.2; N, 20.4.

4 - {*p* - [N,N - Bis(β - chloroethyl)amino]benzalhydrazono} - 6 - chloropyrimidine, m.p. 295–297°, was prepared from 6-chloro-4-hydrazinopyrimidine (9) in 86% yield.

Anal.—Calcd. for $C_{15}H_{16}Cl_3N_5$: C, 48.3; H, 4.3; N, 18.7. Found: C, 48.5; H, 4.3; N, 18.9.

5 - {*p* - [N,N - Bis(β - chloroethyl)amino]benzalhydrazono}uracil, m.p. 225° dec., was prepared from 5-hydrazinouracil hydrochloride (14) in cold water in 74% yield.

Anal.—Calcd. for $C_{15}H_{17}Cl_2N_5O_2$; C, 48.4; H, 4.6; N, 18.8. Found: C, 48.8; H, 4.7; N, 18.7.

5 - {*p* - [N,N - Bis(β - chloroethyl)amino]benzalhydrazonocarbonyl}uracil, m.p. 290° dec., was prepared from 5-uracilcarbohydrazide hydrochloride in 56% yield.

Anal.—Calcd. for $C_{16}H_{17}Cl_2N_5O_3$: C, 48.2; H, 4.3; N, 17.6. Found: C, 48.3; H, 4.5; N, 17.6.

6 - {*p* - [N,N - Bis(β - chloroethyl)amino]benzalhydrazonocarbonyl}uracil, m.p. 182° dec., was prepared from orotic acid hydrazide (15), using dimethylformamide as solvent, in 79% yield.

Anal.—Calcd. for $C_{16}H_{17}Cl_2N_5O_3 \cdot H_2O$: C, 46.1; H, 4.6; N, 16.8. Found: C, 46.0; H, 4.7; N, 16.4.

2 - {*p* - [N,N - Bis(β - chloroethyl)amino]benzalhydrazono}benzothiazole, m.p. 197–199°, was prepared from 2-hydrazinobenzothiazole (16) in 43% yield as light yellow crystals.

Anal.—Calcd. for $C_{18}H_{18}Cl_2N_4S$: C, 55.0; H, 4.5; N, 14.2. Found: C, 55.3; H, 4.8; N, 13.9.

2 - {*p* - [N,N - Bis(β - chloroethyl)amino]benzalhydrazonomethyl}naphthimidazole, m.p. 225° dec., was prepared from 2-(hydrazinomethyl)-naphthimidazole in 56% yield as white crystals.

Anal.—Calcd. for $C_{23}H_{23}Cl_2N_5$: C, 62.7; H, 5.2; N, 15.9. Found: C, 62.3; N, 5.4; N, 16.3.

4 - {*p* - [N,N - Bis(β - chloroethyl)amino]benzalhydrazonosulfonyl}toluene, m.p. 179–181°, was prepared from *p*-toluenesulfonyl hydrazide (17) in 75% yield as light orange crystals.

Anal.—Calcd. for $C_{18}H_{21}Cl_2N_3O_2S$: C, 52.2; H, 5.2; N, 10.2. Found: C, 52.2; H, 5.2; N, 10.0.

Di - {*p* - (N,N - bis(β - chloroethyl)amino]benzal}oxalic carbohydrazone, m.p. 244–245°, was prepared from oxalyl hydrazide (18) in 77% yield as light yellow crystals.

Anal.—Calcd. for $C_{24}H_{28}Cl_4N_6O_2$: C, 50.2; H, 4.9; N, 14.6. Found: C, 50.3; H, 5.2; N, 14.2.

4 - {*p* - [N,N - Bis(β - chloroethyl)amino]benzalhydrazono}benzoic acid, m.p. 226–228°, was prepared from *p*-(hydrazino)benzoic acid (19) in 74% yield.

Anal.—Calcd. for $C_{18}H_{19}Cl_2N_3O_2$: C, 56.9; H, 5.0; N, 11.0. Found: C, 57.1; H, 5.3; N, 10.8.

4 - {*p* - [N,N - Bis(β - chloroethyl)amino]benzal}thiosemicarbazone, m.p. 189–191°, from thiosemicarbazide in 63% yield as light yellow crystals.

Anal.—Calcd. for $C_{12}H_{16}Cl_2N_4S$: C, 45.2; H, 5.0; N, 17.5. Found: C, 45.4; H, 5.1; N, 17.2.

3- Methyl - 6(1′ - methyl - 2′ - {*p* - [N,N - bis(β-chloroethyl)amino]benzal}hydrazono) - 2 - (methylthio)-4(3H)pyrimidinone, m.p. 218°, was prepared from 3 - methyl - 6 - (1′ - methylhydrazino) - 2 - (methylthio) - 4(3H)-pyrimidinone (20) in 80% yield as light brown crystals.

Anal.—Calcd. for $C_{18}H_{23}Cl_2N_5OS$: C, 50.5; H, 5.4; N, 16.4 Found: C, 50.3; H, 5.6; N, 16.8.

3 - Methyl - 6 - (1′ - methyl - 2′ - {*p* - [N,N-bis(β - chloroethyl)amino]benzal}hydrazono)uracil, m.p. 239°, was prepared from 3-methyl-6-(1′-methylhydrazino)uracil (20) in 50% yield.

Anal.—Calcd. for $C_{17}H_{21}Cl_2N_5O_2$: C, 51.3; H, 5.3; N, 17.6. Found: C, 51.3; H, 5.5; N, 17.5.

3-Methyl-6-(1′-methyl-2′-{*p*-[N,N-bis(β-chloroethyl)amino]benzal} - hydrazino) - 5 - (phenylazo) uracil, m.p. 204°, was obtained from 3-methyl-6-(1′-methylhydrazino)-5-(phenylazo)uracil (20) in 48% yield.

Anal.—Calcd. for $C_{23}H_{25}Cl_2N_7O_2$: C, 55.0; H, 5.0; N, 19.5. Found: C, 54.8; H, 4.9; N, 19.7.

The following compounds were prepared by essentially the same procedure except that 5 ml., rather than two drops, of concentrated hydrochloric acid was added to the reaction mixture. As a result, the desired product was isolated as its hydrochloride.

4 - {*p* - [N,N - Bis(β - chloroethyl)amino] benzalhydrazono} - 5 - bromo - 2 - methylthiopyrimidine hydrochloride, m.p. 210–213° dec., was prepared from 5 - bromo - 4 - hydrazino - 2 - methylthiopyrimidine in 56% yield.

Anal.—Calcd. for $C_{16}H_{18}BrCl_2N_5S \cdot HCl$: C, 38.5; H, 3.8; N, 14.1. Found: C, 38.7; H, 4.0; N. 14.5.

2,4 - Di - {*p* - [N,N - bis(β - chloroethyl)amino]-benzalhydrazono}pyrimidine dihydrochloride, m.p. 180° dec., was prepared from 2,4-di(hydrazino)-pyrimidine (21) in 40% yield.

Anal.—Calcd. for $C_{26}H_{30}Cl_4N_8 \cdot 2HCl$: C, 46.8; H, 4.8; N, 16.8. Found: C, 46.5; H, 4.7; N, 16.7.

2,4 - Di - {*p* - [N,N - bis(β - chloroethyl)amino]-benzalhydrazono} - 6 - methylpyrimidine hydrochloride, m.p. 190° dec., was prepared from 2,4-di(hydrazino)-6-methylpyrimidine (22) in 67% yield.

Anal.—Calcd. for $C_{27}H_{32}Cl_4N_8 \cdot HCl$: C, 50.1; H, 5.1; N, 17.3. Found: C, 49.7; H, 5.4; N, 17.3.

5 - Bromo - 4,6 - di{*p* - [N,N - bis(β - chloroethyl)-amino]benzalhydrazono}pyrimidine hydrochloride, m.p. 207° dec. was prepared from 5-bromo-4,6-di-(hydrazino)pyrimidine in 79% yield.

Anal.—Calcd. for $C_{26}H_{29}BrCl_4N_8 \cdot HCl$: C, 43.8; H, 4.4; N, 15.8. Found: C, 43.4; H, 4.8; N, 15.8.

SUMMARY

A number of hydrazono derivatives of *p*-[N,N-bis(β-chloroethyl)amino]benzaldehyde have been synthesized. The compounds have been submitted for general screening at the Cancer Chemotherapy National Service Center.

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By OLE GISVOLD

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PREVIOUS reports (1) on the nature of the chief desglucoglycosides of *Digitalis mertonensis*[1] yielded somewhat inconclusive results. In these investigations both fresh and dried leaves were used together with a number of different extraction conditions and solvents to prepare the primary extracts. Both acetyldigitoxin and digitoxin, when combined, represented the major desglucoglycosides. In one case it was chiefly digitoxin, in another, it was chiefly acetyldigitoxin. It now has been demonstrated that when a primary extract of fresh leaves of *D. mertonensis* is made with 35 per cent methanol and the subsequent purification steps carried out with care, acetyldigitoxin is the chief desglucoglycoside that can be detected and subsequently isolated in good yield. The leaves must be in a healthy condition and free from damaged or partially damaged leaves. Where any amounts of the

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Anal.—Calcd. for $C_{17}H_{21}Cl_2N_5O_2$: C, 51.3; H, 5.3; N, 17.6. Found: C, 51.3; H, 5.5; N, 17.5.

3-Methyl-6-(1′-methyl-2′-{*p*-[N,N-bis(β-chloroethyl)amino]benzal} - hydrazino) - 5 - (phenylazo) uracil, m.p. 204°, was obtained from 3-methyl-6-(1′-methylhydrazino)-5-(phenylazo)uracil (20) in 48% yield.

Anal.—Calcd. for $C_{23}H_{25}Cl_2N_7O_2$: C, 55.0; H, 5.0; N, 19.5. Found: C, 54.8; H, 4.9; N, 19.7.

The following compounds were prepared by essentially the same procedure except that 5 ml., rather than two drops, of concentrated hydrochloric acid was added to the reaction mixture. As a result, the desired product was isolated as its hydrochloride.

4 - {*p* - [N,N - Bis(β - chloroethyl)amino] benzalhydrazono} - 5 - bromo - 2 - methylthiopyrimidine hydrochloride, m.p. 210–213° dec., was prepared from 5 - bromo - 4 - hydrazino - 2 - methylthiopyrimidine in 56% yield.

Anal.—Calcd. for $C_{16}H_{18}BrCl_2N_5S \cdot HCl$: C, 38.5; H, 3.8; N, 14.1. Found: C, 38.7; H, 4.0; N. 14.5.

2,4 - Di - {*p* - [N,N - bis(β - chloroethyl)amino]-benzalhydrazono}pyrimidine dihydrochloride, m.p. 180° dec., was prepared from 2,4-di(hydrazino)-pyrimidine (21) in 40% yield.

Anal.—Calcd. for $C_{26}H_{30}Cl_4N_8 \cdot 2HCl$: C, 46.8; H, 4.8; N, 16.8. Found: C, 46.5; H, 4.7; N, 16.7.

2,4 - Di - {*p* - [N,N - bis(β - chloroethyl)amino]-benzalhydrazono} - 6 - methylpyrimidine hydrochloride, m.p. 190° dec., was prepared from 2,4-di(hydrazino)-6-methylpyrimidine (22) in 67% yield.

Anal.—Calcd. for $C_{27}H_{32}Cl_4N_8 \cdot HCl$: C, 50.1; H, 5.1; N, 17.3. Found: C, 49.7; H, 5.4; N, 17.3.

5 - Bromo - 4,6 - di{*p* - [N,N - bis(β - chloroethyl)-amino]benzalhydrazono}pyrimidine hydrochloride, m.p. 207° dec. was prepared from 5-bromo-4,6-di-(hydrazino)pyrimidine in 79% yield.

Anal.—Calcd. for $C_{26}H_{29}BrCl_4N_8 \cdot HCl$: C, 43.8; H, 4.4; N, 15.8. Found: C, 43.4; H, 4.8; N, 15.8.

SUMMARY

A number of hydrazono derivatives of *p*-[N,N-bis(β-chloroethyl)amino]benzaldehyde have been synthesized. The compounds have been submitted for general screening at the Cancer Chemotherapy National Service Center.

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PREVIOUS reports (1) on the nature of the chief desglucoglycosides of *Digitalis mertonensis*[1] yielded somewhat inconclusive results. In these investigations both fresh and dried leaves were used together with a number of different extraction conditions and solvents to prepare the primary extracts. Both acetyldigitoxin and digitoxin, when combined, represented the major desglucoglycosides. In one case it was chiefly digitoxin, in another, it was chiefly acetyldigitoxin. It now has been demonstrated that when a primary extract of fresh leaves of *D. mertonensis* is made with 35 per cent methanol and the subsequent purification steps carried out with care, acetyldigitoxin is the chief desglucoglycoside that can be detected and subsequently isolated in good yield. The leaves must be in a healthy condition and free from damaged or partially damaged leaves. Where any amounts of the

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latter were present, digitoxin also could be detected. Leaves that had been dried rapidly at 50° in 1958 and 1961 yielded chiefly acetyldigitoxin together with very small amounts of digitoxin. Leaves that had been dried less rapidly in 1958, 1959, and 1960 yielded larger amounts of digitoxin together with acetyldigitoxin. Leaves dried at 70° gave results comparable to those dried rapidly at 50°. Drying at 70° was prompted by the discovery (unpublished data) that when *D. siberica* was dried under these conditions the chief desglucoglycoside was digitoxin, while the chief native glycoside is lanatoside A. In the limited number of conditions used to dry the leaves of *D. mertonensis*, varying amounts of deacetylation occurred. As a continuation of this project other drying conditions shall be studied in order to determine if deacetylation can be completely avoided and also if complete deacetylation can be effected. In the latter case, this would lead to the convenient isolation of digitoxin which occurs in about twice the amount that occurs in *D. purpurea* grown in Minnesota.

The chief objective of this report is to describe the convenient isolation of acetyldigitoxin from *D. mertonensis* and *D. siberica*. These techniques also were applied to the isolation of digitoxin from *D. purpurea* with marked success. Aqueous methanol 35 per cent was chosen to prepare an initial (primary) extract in all cases. The desired desglucoglysides readily could be extracted from this initial extract with methyl isoamyl ketone (MIAK). The latter solvent was chosen also because it removed a minimum amount of undesirable substances, can be recovered in good yield, and is not as hazardous as some organic solvents. The initial extract from dried leaves of *D. mertonensis* that had been stored for several years contained degradation products of chlorophyll that carried over into the MIAK extracts and subsequently proved somewhat troublesome. This was not true with freshly dried leaves. Initial extraction of the dry leaves with benzene[2] did not prove advantageous. Extraction of the initial extract with isopropyl ether readily removed these troublesome pigments together with small amounts of acetyldigitoxin and digitoxin. This step may or may not prove advantageous, depending upon the amount of colored degradation products that might be encountered in the primary extract. In the case of carefully collected fresh leaves or carefully dried leaves that are soon used, the extraction with isopropyl ether is not necessary.

[2] With the hope that the benzene soluble degradation products of chlorophyll could be eliminated.

In an attempt to shorten the isolation procedure, the MIAK extract from the primary extract was extracted with aqueous alkali to remove certain yellow pigments. This modification did not lead to deacetylation of acetyldigitoxin if the aqueous alkaline phase was rapidly separated from the MIAK phase. However, fewer pigments were removed by this technique than when they were removed from a mixture of ether-methylene dichloride (3:1).

EXPERIMENTAL

The details of the paper chromatographic techniques used in these studies have been described previously (2). Both solvent systems I and II were used for the development of the paper chromatograms. The Raymond reagent, Kedde reagent, or Jensen's trichloroacetic acid reagent (3) were used to detect the position of the glycosides on the paper. The Raymond reagent readily detected digitoxin and acetyldigitoxin, however, gitoxin was difficult to detect by this reagent. This no doubt was due to the poor solubility of gitoxin. The Kedde reagent readily detected gitoxin and when equal amounts of digitoxin and gitoxin were spotted on paper and then treated with the Kedde reagent, the color intensities were comparable in both cases.

THE ISOLATION OF ACETYL DIGITOXIN

The leaves of *D. mertonensis* collected in the fall of 1961 were rapidly dried at 50° with aid of a fan. The powdered leaves, 250 Gm., were macerated overnight with 1 L. of 35% methanol. The next day they were packed in a percolator, percolated slowly with 35% methanol, and 1500 ml. of percolate was collected. This initial extract then was extracted with three successive portions of 200 ml. each of methylisoamyl ketone by means of a separatory funnel. Some emulsions were encountered that could be broken with anhydrous sodium sulfate. This was the only step that proved somewhat troublesome. Some of the yellow pigments could be extracted from this MIAK extract with aqueous sodium or potassium hydroxide 5%. The MIAK was removed under vacuum (water pump) at 38 to 45°. The dark-colored amorphous mass was digested with methylene chloride which partially dissolved in this solvent. The insoluble yellow-brown solid was removed either by filtration or by use of a centrifuge at 5000 r.p.m., using polypropylene test tubes. This colored insoluble material contained pigments (flavones) that have been partially characterized and will be the subject of a future report. The methylene chloride solution (40 ml.) was diluted with 120 ml. of ether and subsequently extracted with two 10-ml. portions of 5% aqueous potassium hydroxide in order to remove certain yellow flavones and related pigments. These pigments, together with the aforementioned methylene chloride insoluble pigments, which also are alkali soluble, are usually removed by lead acetate or basic lead acetate. We previously have shown that basic lead acetate can effect some deacetylation of the acetyl glycosides. Also, the

use of these reagents for clarification purposes, pigment removal, *etc.*, sometimes results in manipulative techniques that might better be replaced by simpler techniques that accomplish the same results. After washing once with water, the organic solution then was washed with 10 ml. of 20% of sodium phosphate monobasic and then once again with water. Sodium phosphate monobasic is an excellent way to remove the excess alkali in that it is much superior to acetic acid and permits the ready removal by washing of any excess of the acidifying agent. The removal of any excess acetic acid is most troublesome; for at this stage, if the ether methylene chloride solution is allowed to stand overnight, the separation of small amounts of glycosides takes place. However, the solvent was directly removed by distillation until a concentrate of about 20 ml. was obtained. The concentrate was impregnated on filter cell 4 Gm. and dried. The mixture was placed in a sintered-glass funnel and eluted slowly with 150 ml. of a mixture of 60 parts skellysolve B and 40 parts benzene. This solvent mixture removed some nonphenolic pigments and other resin-like substances that have not been characterized. The mixture then was eluted with 150–200 ml. of anhydrous ether. Upon concentration of the ether eluate to a small volume, acetyldigitoxin crystallized out directly in the distillation flask. The glycoside was collected by means of a sintered-glass filter. The filtrate was diluted with an equal volume of skellysolve B (hexane) and additional amounts of glycoside separated out that was collected by filtration. When the paper was examined chromatographically, the above two fractions contained chiefly acetyldigitoxin. The procedure in subsequent isolations was shortened by adding the skellysolve B directly to the ether concentrate and the total glycosides obtained by one filtration. Concentration of the ether–skellysolve filtrate to remove about half the ether caused the separation of further small quantities of acetyldigitoxin together with some green pigments. The filter cell finally was extracted with methylene dichloride to yield an additional small amount of glycosides which could have been extracted very slowly by additional quantities of ether. Of the solvents tested, ether gave the most satisfactory results and gave an initial glycoside preparation that had a very high glycoside concentration consisting almost entirely of acetyldigitoxin.

Acetyldigitoxin also can be eluted from the filter cell mixture by isopropyl ether, and upon concentration of this eluate the glycosides separated out as when ether was used.

When 250-Gm. samples of dry *D. mertonensis* were assayed by the above described techniques, values of the order of 0.55 Gm. of acetyldigitoxin were obtained, or 2.10 Gm. per Kg.

In the case of dried leaves in which some deacetylation had taken place during the drying, the same approximate weights of glycoside were obtained that were mixtures of digitoxin and acetyldigitoxin.

When fresh leaves of *D. mertonensis* were used, the only variation in the above techniques was the disintegration of the fresh with aqueous methanol of such a concentration that the final extract was 35%. With fresh leaves, as previously stated, one can be assured of very little or no deacetylation.

Although no effort was made to isolate or determine the acetylgitoxin or gitoxin fractions, all evidence obtained to date indicates that very small amounts of digilanide B are present in *D. mertonensis*. Contrast this with the large amounts of gitoxin (from purpurea glycoside B) that occur in *D. purpurea* grown in Minnesota.

The same techniques that were used for the isolation of acetyldigitoxin from *D. mertonensis* were applied to both the fresh and dried leaves of *D. siberica*. The results were essentially the same as those obtained with *D. mertonensis*, however, considerably fewer pigments were encountered, especially the flavone type. Quantitative studies will be carried out at a later date and reported.

The Isolation of Digitoxin from *D. purpurea*.—The isolation of digitoxin from *D. purpurea* was accomplished with ease by the same techniques used to isolate acetyldigitoxin from *D. mertonensis*. The gitoxin separated from the methylene dichloride ether solution both before and after concentration and very little gitoxin was encountered in the final digitoxin preparation.

When 250 Gm. samples of dry *D. purpurea* were assayed by the above described techniques, values of the order of 0.225 Gm. of digitoxin were obtained, or 0.90 Gm. per Kg. This is less than half of that obtainable from *D. mertonensis*.

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ERRATUM

In the paper titled "Rheology of Thixotropic Montmorillonite Dispersions II. Kinetics of Structural Recovery" (1), the legend in the caption for Fig. 1 should read: △, 2 weeks; ●, 6 weeks; ○, 11 weeks; and □, 17 weeks. In addition, the reference to Fig. 3 in line 4, paragraph 5, column two, at page 953 should be a reference to Fig. 1.

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Preliminary Report on the Comparative Stability of Certified Colorants with Lactose in Aqueous Solution

By CHARLES A. BROWNLEY, JR., and LEON LACHMAN

The influence of conventional lactose and spray-processed lactose and their primary hydrolysis products (*d*-glucose and *d*-galactose) on the stability of various certified colorants has been investigated. Spectrophotometric and paper partition chromatographic examination indicated that in aqueous buffered solutions (pH 6.6 to 6.8), FD&C Red No. 4, FD&C Yellow No. 5, FD&C Green No. 3, and FD&C Blue No. 1 are relatively stable when exposed to exaggerated lighting and temperature in the presence of these sugars. FD&C Blue No. 2 was found to be very unstable, particularly when exposed to light, and the sugars significantly accelerate the decomposition of this color. The decomposition of FD&C Blue No. 2 appears to proceed by reduction to a semiquinone followed by oxidation. There appears to be some evidence to indicate molecular changes in the lactose when stored at high temperature. These changes appear to interfere with the spectrophotometric analysis of colorants which absorb between 228 mμ and 284 mμ.

THE INTRODUCTION of spray-drying procedures for the manufacture of alpha lactose monohydrate and the subsequent acceptance of this sugar by the official compendia afforded the pharmaceutical tablet developer with a pharmaceutical excipient exhibiting a number of advantages. Among these are flow and compaction properties unknown with conventionally manufactured lactose. The use of spray-dried lactose permits the economical production of tablets by direct compaction, a process which consists of only two steps—powder blending and compression. Through this method of preparation it is possible to formulate tablets of heat and moisture-sensitive ingredients in a simple manner and with enhanced stability.

Efforts to produce colored tablets by direct compaction through the use of blended dye triturates, lakes, or precolored disintegrants or lubricants with spray-processed lactose were not completely successful and were rather costly. Through the use of colored spray-dried lactose (the lactose being colored during the spray-drying operation), the preparation of colored tablets by direct compaction was made possible. The colored spray-dried lactose exhibits similar flow and compaction properties as the uncolored material. The preparation of colored spray-dried lactose was initiated in 1958 by Brownley and Reger (1).

The initial batches were manufactured with certified FD&C Blue No. 1, FD&C Yellow No. 5, FD&C Green No. 3, FD&C Orange No. 3, and the formerly certified FD&C Red No. 1. The quantities of colorants used varied from 0.008 to 0.05%. Preliminary investigation indicated that fading and color changes occurred in some of these lactoses. As examples, the colored lactose containing 0.05% FD&C Yellow No. 5 changed during normal ambient storage from a golden yellow to a dull yellowish-tan. The product containing 0.008% FD&C Red No. 1 varied in color from light to dark pink within a particular batch of lactose. These adverse changes, however, were not evident in colored lactose granules prepared with conventional lactose. These granules had been prepared by customary wet granulating techniques in the same dye concentration as in the colored spray-dried lactoses.

A possible explanation for the difference in color stability for the spray-dried and conventional lactose can be the presence of higher concentrations of lactose's hydrolysis products in the spray-dried lactose, as compared to the conventional lactose. It has been shown by Jenness and Patton (2), Weisberg (3), and Whittier (4) that heat differences during manufacture can cause appreciable difference in the extent of lactose hydrolysis. When analyzing the two types of lactose for other sugars according to U.S.P. methods, results indicated that some batches of spray-dried lactose contained two to four times as much other sugars as found in conventional lactose. The other sugars were identified as being the primary hydrolysis products of lactose, namely, D-glucose and D-galactose. These monosaccharides possess 30% greater reducing power than pure lactose (5). Therefore, it would be expected for the spray-dried

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lactose to have a more deleterious effect on colorants susceptible to reduction reactions than conventional lactose.

Although there has been an increase in recent years in the number of investigations concerned with stability of colorants used in pharmaceutical dosage forms (6–10), these studies did not consider the relative stability of spray-dried and conventionally processed lactose with certified dyes. Since the oxidation–reduction interreactions between dyes and pharmaceutical agents are a serious problem for the pharmaceutical industry, it seemed desirable to evaluate the relative stability of spray-dried lactose *vs.* conventional lactose with several commonly used certified colors. In addition, it was desirable to discern the exact influence of lactose's primary hydrolysis products on the stability of these colorants.

In this investigation, the chemical stability of dye-sugar mixtures was examined in aqueous solutions. Five dyes representing three of the major chemical categories were selected for study. The dyes and sugars were studied at 0.15 and 5% concentrations, respectively, in aqueous solutions buffered at pH 6.6 to 6.8. This buffer system was chosen in order to maintain a constant hydrogen ion concentration as well as to approximate the pH of a 0.15% aqueous solution of the majority of dyes.

EXPERIMENTAL

Materials Used.—Conventionally processed lactose U.S.P. (Avoset Co.); spray-processed lactose U.S.P. (Western Condensing Co.); dextrose U.S.P. (D-glucose); D-galactose (Matheson Coleman and Bell); FD&C Red No. 4 (monoazo); FD&C Blue No. 1 and FD&C Green No. 3 (triphenylmethane); FD&C Yellow No. 5 (pyrazolone); FD&C Blue No. 2 (indigoid) (H. Kohnstamn Co.); ethyl acetate and pyridine (Baker Chemical Co.); benzidine reagent (Matheson Coleman and Bell); Sorensen phosphate buffer solution at pH 6.6 to 6.8.

Equipment.—Beckman recording spectrophotometer, model DK-1; Beckman Zeromatic pH meter; light stability cabinet exhibiting intensified illumination as described in a previous publication (11); precision scientific oven maintained at 60° ± 1°; Whatman's No. 1 chromatographic paper supplied in sheets 18.25 inches × 22.5 inches; microliter pipets; chromatography jars, 18 inches high × 12 inches in diameter, including complete setup for descending development; spray bottles; ultraviolet lamp, long wave (3660 Å).

Procedure for Spectrophotometric Analysis.—Aqueous buffer solutions containing 0.15% dye, 0.15% dye + lactose, 0.15% dye + D-glucose and 0.15% dye + D-galactose were prepared and investigated for colorant stability. These solutions were filtered through sintered glass funnels and filled into 10-ml. clear neutraglas color-break ampuls. The ampuls were then sealed on the Popper HI laboratory ampul sealer. Each batch of ampuls was divided into three groups and stored as follows: Group one was stored at room temperature in light-resistant cardboard containers; group two was stored under intensified illumination in the light stability cabinet; group three was stored in light-resistant cardboard containers at 60° ± 1°. At designated time intervals, individual ampuls were removed and assayed spectrophotometrically by running a complete absorption spectra in the visible and ultraviolet regions. Absorbance was measured at the wavelengths indicated in Table I.

TABLE I.—TEST CONDITIONS USED FOR SPECTROPHOTOMETRIC ANALYSIS

	Absorption Maximum[a]		
	Visible mμ	Uultraviolet mμ	pH of Solution[b]
FD&C Red No. 4	505	303	6.65
FD&C Yellow No. 5	430	257	6.65
FD&C Green No. 3	628	302	6.70
FD&C Blue No. 1	625	307	6.80
FD&C Blue No. 2	617	285	6.72

[a] Beckman recording spectrophotometer, model DK-1.
[b] Beckman Zeromatic pH meter.

Procedure for Paper Partition Chromatography.—The solvent system was prepared by mixing 400 ml. of ethylacetate, 200 ml. of pyridine, and 400 ml. of water in a separatory funnel, shaken and allowed to separate. The lower phase was used to saturate the chamber. The upper phase was used to develop the chromatograms.

The chromatographic jars, used for descending chromatography, were lined with filter paper. The lower phase was placed in the bottom of the chamber with the liner dipping into the solvent. The upper phase was placed in the solvent troughs. The chamber was then allowed to equilibrate for 16 hours.

Points for the application of the sample were marked 3 cm. apart on a line 6.5 cm. from the top of Whatman's No. 1 paper cut to 46-cm. lengths. The samples and standards were dissolved in an appropriate solvent and the samples were spotted on the chromatograms in concentrations of 100 to 500 mcg. The size of the spot should be approximately 5 mm. in diameter. This was accomplished by repeated spotting and drying of the solvent. The chromats were placed into the troughs and developed for approximately 7 hours. The developed chromats were then air dried and sprayed with a benzidine reagent prepared according to Bacon and Edelman (12).

The chromats were then heated in an oven for 15 min. at 90°. The reducing sugars appeared as

TABLE II.—R_f VALUES OF DYES AND SUGARS

Compound	R_f Values[a]
Lactose	0.25
D-Glucose	0.35
D-Galactose	0.35
FD&C Red No. 4	0.55
FD&C Yellow No. 5	0.20
FD&C Blue No. 1	0.60
FD&C Blue No. 2	0.50
FD&C Green No. 3	0.47

[a] Solvent: ethyl acetate-pyridine-water 2:1:2 v/v.

brown spots. The sensitivity was increased by viewing under an ultraviolet lamp. Table II shows the R_f values for the sugars and dyes used in this study.

The use of this ethyl acetate-pyridine-water system for paper partition chromatography of colorants and sugars has the advantage over other previously reported systems for use with carbohydrates (22) in that good separations can be obtained in a shorter period of time.

RESULTS AND DISCUSSION

The influence of conventional lactose and spray-processed lactose and their primary hydrolysis products (D-glucose and D-galactose) on the stability of FD&C Red No. 4, FD&C Yellow No. 5, FD&C Green No. 3 and FD&C Blue No. 1 and No. 2 in solution has been investigated at several storage conditions.

FD&C Red No. 4.—The data presented in Tables III and IV indicate that FD&C Red No. 4 is reasonably stable equally in the presence of spray-processed lactose, conventionally processed lactose and lactose's primary hydrolysis products, D-glucose and D-galactose, at these conditions of testing. A change occurred in the absorption maximum for the buffered dye solutions but no change was seen in the dye-sugar solutions. This change was more pronounced in the visible than the ultraviolet range. The presence of the reducing sugars showed an apparent stabilizing effect on the dye as evidenced particularly by the data showing the influence of light. This improved stability is believed to be due to preferential reduction of the lactose instead of the dye.

TABLE III.—THE EFFECT OF LACTOSE, *d*-GLUCOSE, AND *d*-GALACTOSE ON THE ABSORBANCE OF FD&C RED NO. 4 AT 505 Mμ

FD&C Red No. 4–0.15% in	Zero Time	126 Days RT	28 Days 60°	49 Days E.L.[a]
0.15 *M* Phosphate buffer solution(pH 6.6)	0.470	0.465	0.460	0.422
5% Lactose U.S.P. (regular) solution	0.455	0.458	0.465	0.450
5% Lactose U.S.P. (spray-processed) solution	0.455	0.455	0.460	0.445
5% D-Glucose solution	0.470	0.459	0.465	0.460
5% D-Galactose solution	0.455	0.460	0.465	0.455

[a] Exaggerated lighting.

TABLE IV.—THE EFFECT OF LACTOSE, *d*-GLUCOSE AND *d*-GALACTOSE ON THE ABSORBANCE OF FD&C RED NO. 4 AT 303 Mμ

FD&C Red No. 4–0.15% in	Zero Time	126 Days RT	28 Days 60°	49 Days E.L.[a]
0.15 *M* Phosphate buffer solution (pH 6.6)	0.328	0.319	0.322	0.310
5% Lactose U.S.P. (regular) solution	0.319	0.315	0.323	0.320
5% Lactose U.S.P. (spray-processed) solution	0.322	0.313	0.320	0.323
5% D-Glucose solution	0.321	0.316	0.323	0.323
5% D-Galactose solution	0.320	0.318	0.323	0.325

[a] Exaggerated lighting.

This apparent stability of the dye-sugar solution was further shown by paper partition chromatography examination of the FD&C Red No. 4–sugar solutions after storage, as evidenced by no development of R_f values different from those obtained initially.

These results are in keeping with the recent findings of Swartz, *et al.* (13), in which tablets buffered to pH 7 containing FD&C Red No. 4 exhibited excellent chemical stability after storage for 20 days at 80°.

FD&C Yellow No. 5.—The effect of various sugars on the thermal and light stability of FD&C Yellow No. 5 in solution is summarized in Tables V and VI. The absorbance values indicate apparent stability of FD&C Yellow No. 5 with spray-processed lactose, conventionally processed lactose, and the hydrolysis products, D-glucose and D-galactose.

TABLE V.—THE EFFECT OF LACTOSE, D-GLUCOSE, AND D-GALACTOSE ON THE ABSORBANCE OF FD&C YELLOW NO. 5 AT 430 Mμ

FD&C Yellow No. 5–0.15% in	Zero Time	105 Days RT	105 Days 60°	105 Days E.L.[a]
Distilled water	0.370	0.350	0.345	0.352
0.15 *M* Phosphate buffer solution (pH 6.6)	0.365	0.354	0.353	0.350
5% Lactose U.S.P. (regular) solution	0.360	0.350	0.346	0.342
5% Lactose U.S.P. (spray-processed) solution	0.365	0.348	0.341	0.335
5% D-Glucose solution	0.370	0.350	0.348	0.342
5% D-Galactose solution	0.370	0.350	0.348	0.342

[a] Exaggerated lighting.

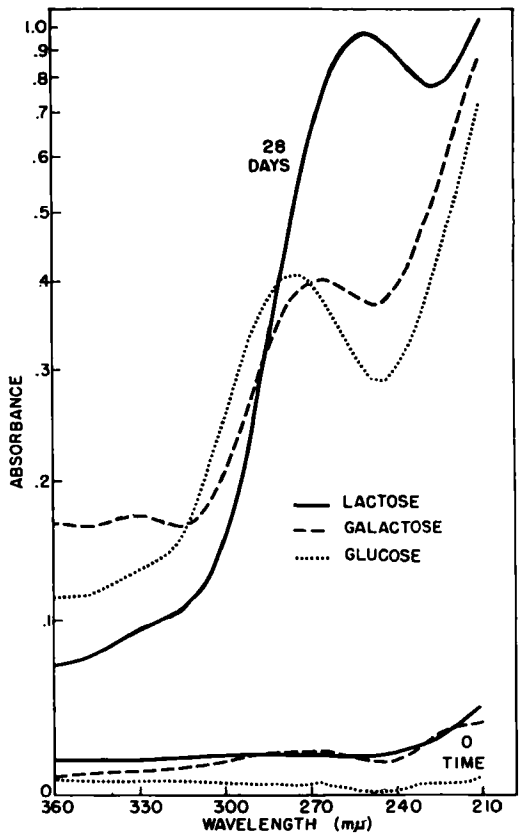

Fig. 1.—A plot of the ultraviolet absorption spectra of 5% solutions of lactose, D-glucose, and D-galactose indicating a change in the spectra after 28 days storage at 60°.

TABLE VI.—THE EFFECT OF LACTOSE, D-GLUCOSE, AND D-GALACTOSE ON THE ABSORBANCE OF FD&C YELLOW No. 5 AT 257 mμ

FD&C Yellow No. 5–0.15% in	Zero Time	105 Days RT	105 Days 60°	105 Days E.L.[a]
Distilled water	0.358	0.342	0.338	0.340
0.15 M Phosphate buffer solution (pH 6.6)	0.359	0.342	0.345	0.335
5% Lactose U.S.P. (regular) solution	0.350	0.335	0.349	0.330
5% Lactose U.S.P. (spray-processed) solution	0.355	0.335	0.348	0.340
5% D-Glucose solution	0.358	0.338	0.360	0.343
5% D-Galactose solution	0.358	0.338	0.360	0.343

[a] Exaggerated lighting.

A diminution in absorbance is seen for the dye-sugar solutions exposed to light. This is in keeping with the report by Lachman, *et al.* (14), who have shown that a flattening as well as a hypsochromic shift of the absorption maximum occurs for FD&C Yellow No. 5 when this colorant is exposed to exaggerated illumination in tablet formulations.

The dye-sugar solutions, after storage at 60°, failed to show the decrease in absorbance at 257 mμ as seen for the room temperature and light samples stored for the same period of time. This may be explained by the changes which take place in the sugar molecule when stored at 60° resulting in high absorption tendencies in the wavelength region of 228–284 mμ as shown in Fig. 1. Since the analysis for FD&C Yellow No. 5 was performed within this range, the results obtained would be expected to be higher than the true values because the absorbance due to the sugars is now playing a role. The tendency of the sugars to appear to develop absorption properties in this wavelength region is being further investigated.

Upon examining the paper chromatograms of the solutions of dye and dye-plus-sugars after storage, no change in R_f values was noticed. This further illustrates the apparent stability of FD&C Yellow No. 5 under the conditions of study. Exposure to exaggerated lighting, however, should be avoided.

FD&C Green No. 3.—The data presented in Tables VII and VIII show FD&C Green No. 3 to be essentially stable in the presence of lactose and its hydrolysis products. Paper partition chromatographic examination of these solutions stored for the same periods of time substantiated this effect. From the paper chromatograms shown in Fig. 2, it is indicated that four impurities (R_f values 0.15, 0.35, 0.65, and 0.75), initially present in the dye, subsequently disappeared upon storage. A possible explanation for this is that the impurities were over-

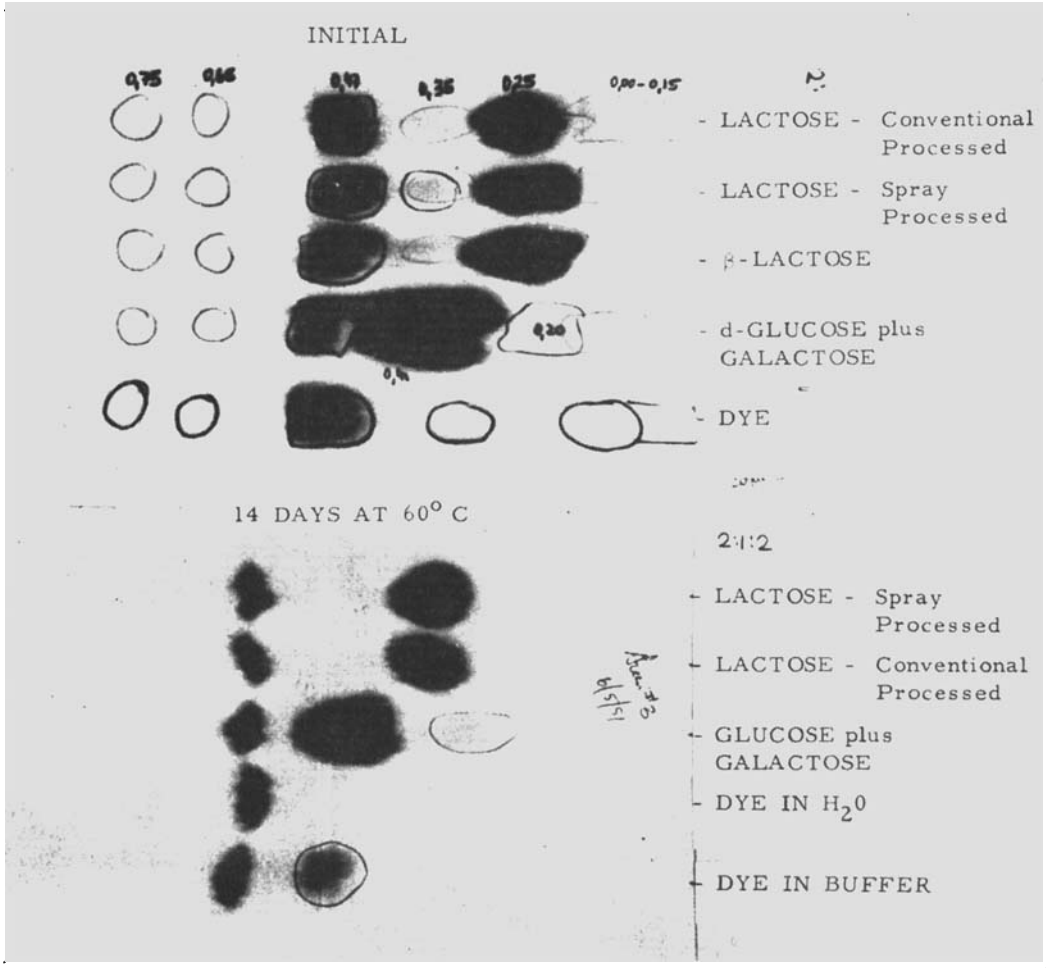

Fig. 2.—Paper chromatograms of FD&C Green No. 3 and FD&C Green No. 3 plus sugar solutions indicating change in R_f value after 14 days at 60°.

TABLE VII.—THE EFFECT OF LACTOSE, D-GLUCOSE, AND D-GALACTOSE ON THE ABSORBANCE OF FD&C GREEN NO. 3 AT 628 mμ

FD&C Green No. 3–0.15% in	Zero Time	150 Days RT	150 Days 60°	92 Days E.L.[a]
Distilled water	0.420	0.399	0.406	0.418
0.15 *M* Phosphate buffer solution (pH 6.6)	0.420	0.395	0.405	0.425
5% Lactose U.S.P. (regular) solution	0.410	0.400	0.418	0.422
5% Lactose U.S.P. (spray-processed) solution	0.420	0.403	0.418	0.436
5% D-Glucose solution	0.420	0.403	0.418	0.423
5% D-Galactose solution	0.420	0.403	0.418	0.423

[a] Exaggerated lighting.

TABLE VIII.—THE EFFECT OF LACTOSE, D-GLUCOSE, AND D-GALACTOSE ON THE ABSORBANCE OF FD&C GREEN NO. 3 AT 302 mμ

FD&C Green No. 3–0.15% in	Zero Time	150 Days RT	150 Days 60°	92 Days E.L.[a]
Distilled water	0.264	0.253	0.260	0.258
0.15 *M* Phosphate buffer solution (pH 6.6)	0.260	0.259	0.261	0.264
5% Lactose U.S.P. (regular) solution	0.255	0.260	0.280	0.263
5% Lactose U.S.P. (spray-processed) solution	0.260	0.259	0.279	0.267
5% D-Glucose solution	0.262	0.259	0.287	0.270
5% D-Galactose solution	0.262	0.259	0.282	0.270

[a] Exaggerated lighting.

oxidized colors. This postulation is in keeping with the spectrophotometric and partition chromatographic findings of Jones, *et al.* (15). Their analysis of various samples of commercial triphenylmethane colors accounted for an average of 94% of the material as the sum of pure dye, leuco compound, volatile material, and inorganic salts. These investigators concluded that the remaining material was chiefly overoxidized color.

FD&C Blue No. 1.—Aqueous solutions of FD&C Blue No. 1 with the various sugars were stored similarly to the FD&C Green No. 3 and no change in absorbance value at 625 mμ was noticed to occur with this dye in the presence of the sugars stored at room temperature and 60° as presented in Table IX.

TABLE IX.—THE EFFECT OF LACTOSE, D-GLUCOSE, AND D-GALACTOSE ON THE ABSORBANCE OF FD&C BLUE NO. 1 AT 625 mμ

FD&C Blue No. 1–0.15% in	Zero Time	90 Days RT	90 Days 60°	90 Days E.L.[a]
Distilled water	0.870	0.870	0.860	0.860
0.15 *M* Phosphate buffer solution (pH 6.6)	0.870	0.870	0.860	0.850
5% Lactose U.S.P. (regular) solution	0.875	0.870	0.870	0.870
5% Lactose U.S.P. (spray-processed) solution	0.870	0.870	0.870	0.830
5% D-Glucose solution	0.875	0.870	0.870	0.810
5% D-Galactose solution	0.870	0.870	0.870	0.710

[a] Exaggerated lighting.

However, at 625 mμ, the spectrophotometric data indicated a decrease in absorbance for the FD&C Blue No. 1 in solution with spray-processed lactose, D-glucose, and D-galactose exposed to exaggerated lighting for 90 days. The data indicated that the effect on the absorbance was greatest with the D-galactose.

No absorbance at 307 mμ was observed for this dye in the presence of the sugars at 60° as illustrated in Table X. The absorption maximum was found to

TABLE X.—THE EFFECT OF LACTOSE, D-GLUCOSE, AND D-GALACTOSE ON THE ABSORBANCE OF FD&C BLUE NO. 1 AT 307 mμ

FD&C Blue No. 1–0.15% in	Zero Time	90 Days RT	90 Days 60°	90 Days E.L.[a]
Distilled water	0.115	0.118	0.113	0.107
0.15 *M* Phosphate buffer solution (pH 6.6)	0.114	0.113	0.113	0.107
5% Lactose U.S.P. (regular) solution	0.117	0.113	...	0.113
5% Lactose U.S.P. (spray-processed) solution	0.115	0.110	...	0.110
5% D-Glucose solution	0.115	0.113	...	0.110
5% D-Galactose solution	0.117	0.118	...	0.105

[a] Exaggerated lighting.

shift from 307 to 280 mμ as shown in Fig. 3. This effect can possibly be explained by the sugar molecule being thermally degraded producing products which absorb at 280 mμ as previously shown in Fig. 1.

No changes were detected in the FD&C Blue No. 1 solutions stored at room temperature and 60° by paper partition chromatographic procedures. There was some indication of dye loss in the samples kept for 90 days in the light cabinet.

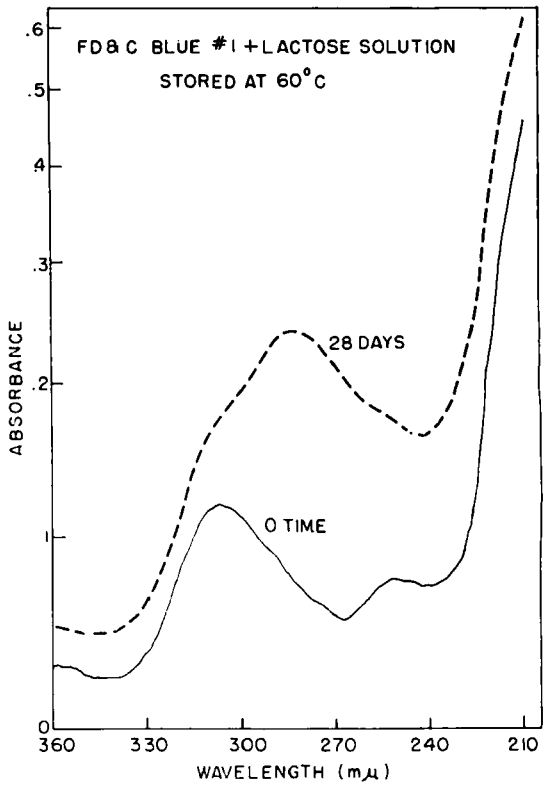

Fig. 3.—Plot showing the influence of lactose on the ultraviolet absorption maximum of FD&C Blue No. 1 after 28 days' storage at 60°.

FD&C Blue No. 2.—Although studies with the dye FD&C Blue No. 2 are still in progress, it is felt worth while to mention certain observations obtained thus far. The preliminary data indicated that lactose, D-glucose, and D-galactose were very deleterious to the stability of this colorant and that the monosaccharides were more destructive than the unhydrolyzed disaccharide. The absorption data obtained from these conditions of testing were very erratic and did not permit kinetic interpretation. Figure 4 shows the spectra changes for FD&C Blue No. 2 in the presence of D-glucose after 3, 6, and 10 days exposure to light. For the 10-day sample, two different spectra (A and B) were obtained for the same sample taken within minutes of one another. Paper partition chromatographic examination of the aqueous buffered solutions of FD&C Blue No. 1 and FD&C Blue No. 2 plus the sugars further substantiated the findings of instability for this colorant. The initial R_f values for FD&C Blue No. 2 at 0.50, and 0.60 for trace impurities, diminished rapidly until no dye was visible after 14 days of heat and light storage (Fig. 5). After 12 weeks, no dye was detected in the room temperature sample. It was observed that two new R_f values appeared at 0.16 and 0.35, which probably indicated the decomposition products of the dye.

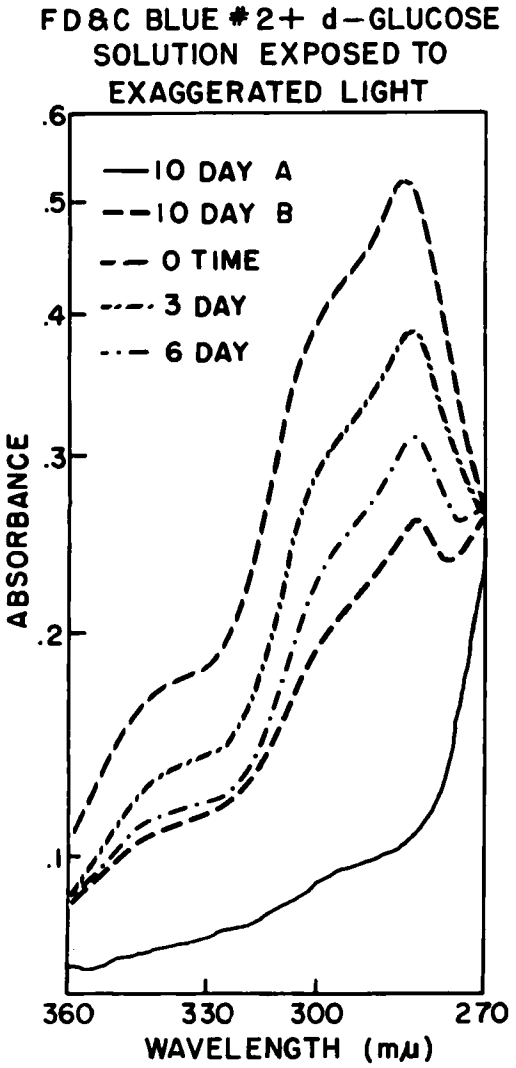

Fig. 4.—A plot of the ultraviolet absorption spectra of FD&C Blue No. 2 indicating changes in the presence of D-glucose after varying periods of storage at 60°.

The decomposition of this indigo disulfonate dye has been studied by several investigators. Scott, *et al.* (8), reporting on the accelerated color loss of certified dyes in the presence of nonionic surfactants, showed that the rate of fading of FD&C Blue No. 2 in the presence or absence of Pluronic F68 follows pseudo first-order kinetics. Kuramoto, *et al.* (7), evaluated the influence of several pharmaceutical materials on the rate of fading of FD&C Blue No. 2 in aqueous solution at pH 6.64 and found that sugars, such as dextrose, lactose, and sucrose, increased the rate of fading of this dye. These investigators postulated that the decomposition of FD&C Blue No. 2 took place by reduction *via* a semiquinone formation to a colorless leuco compound.

Preisler, *et al.* (16), investigated the oxidation–reduction potentials of the indigo sulfonates and their reduced forms and their absorption coefficients in alkaline buffers. These investigators observed that "when indigo in alkaline alcoholic solution or indigo disulfonate in aqueous buffers of about pH 11.0 to 12.5 are reduced by the gradual addition of reducing agent, a red intermediate color appears between the initial blue (or green) of the fully oxidized and the yellow of the reduced compound; on reoxidation the red color appears in the reverse color sequence. Outside this pH zone, the red color is not observed; in less alkaline solutions of the sulfonates, the color changes from blue, through the green of the mixture, to yellow; in more strongly alkaline solutions, the initial color is a yellow, which on reduction changes to a lighter yellow." The data collected by Preisler established conclusively that the red intermediate color was due to a semiquinone formation.

In the present study, a similar reduction pattern was observed. A red color developed after 6 days at 60° in the FD&C Blue No. 2—D-glucose solution between the initial blue and the final yellow color of the faded solution. This red color lasted for only a short period of time. The appearance of this red intermediate at the lower pH's of this study indicated that the semiquinone formation was possible under less alkaline conditions than those observed by Preisler and his co-workers. These data tended to support the postulation of Kuramoto, *et al.*, that reduction to a semiquinone formation occurred in the decomposition of this certified color.

Preliminary results in this investigation indicated that when the sealed ampuls of FD&C Blue No. 2 plus the sugar solutions were exposed to atmospheric conditions, the green-yellow color of the apparently reduced compound changed to a pale blue. On further exposure, the blue color of the solution faded to a pale yellow. The solution at this time was observed to be strongly fluorescent when viewed under ultraviolet light. These changes appeared to indicate that the decomposition of FD&C Blue No. 2 was not solely one of complete reduction to the leuco compounds, but that the mechanism of fading of this indigo disulfonate dye could possibly be reduction followed by oxidation. Inskeep and Kretlow (17)

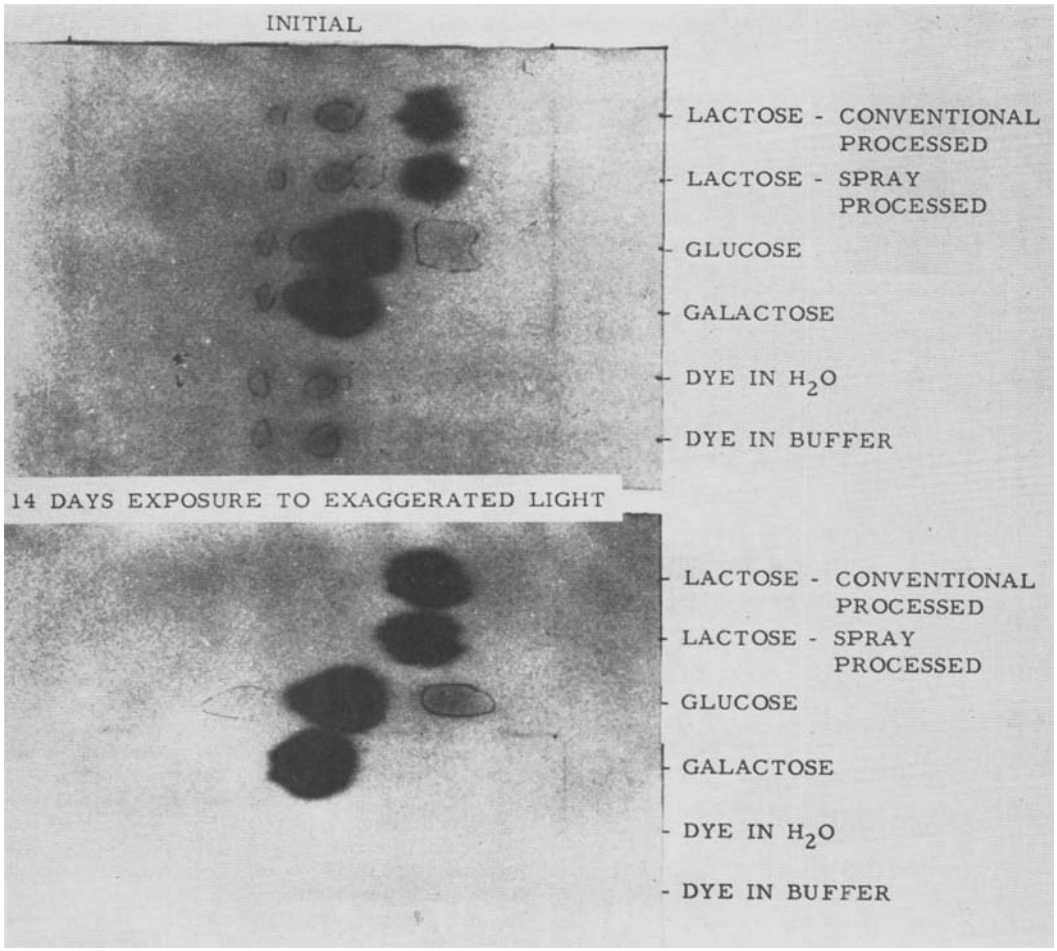

Fig. 5.—Chromatograms showing the R_f values for FD&C Blue No. 2 initially and their absence after 14 days' exposure to exaggerated lighting.

observed that although a common property of dyestuffs is their ability to take up hydrogen with the formation of colorless compounds, the leuco compounds so formed from indigo dyes were oxidized by air.

Jones, *et al.* (18), studied the photodecomposition of dilute solutions of FD&C Blue No. 2 exposed to ordinary and diffuse laboratory light. Spectrophotometric examination showed that the chief component was isatinsulfonic acid. When viewed under ultraviolet light, the faded solutions exhibited a bluish fluorescence characteristic of the sulfonated anthranilic acids. These experiments showed that the fading of solutions of FD&C Blue No. 2 was due almost entirely to the oxidation of the colorant to isatinsulfonic acid and finally to sulfonated anthranilic acid. In alkaline solution, these investigators observed that the indigo disulfonate is converted to disulfonated crysanilic acid which is oxidized by potassium persulfate in acid solution to approximately equal amounts of isatinsulfonic acid and sulfonated anthranilic acid.

That oxidative decomposition was more destructive to this colorant than reduction is stated in the review made by Desai and Giles (19). Hibbert (20) was able to isolate a small quantity of isatin from samples of indigo-dyed cotton cloth which had been faded by exposure to sunlight or to the light from a carbon arc. Zuckerman (21) also reported that FD&C Blue No. 2 had the poorest light resistance of all the certified colors and because of its structure exhibited the best resistance to reducing agents.

SUMMARY AND CONCLUSIONS

In this study the influence of lactose, D-glucose, and D-galactose on the stability of five certified dyes has been investigated. From the results obtained the following conclusions can be drawn.

1. In aqueous solutions buffered to pH 6.6 to 6.8, FD&C Red No. 4, FD&C Yellow No. 5, FD&C Green No. 3, and FD&C Blue No. 1 are relatively stable upon exposure to exaggerated lighting and temperature in the presence of spray-dried and conventional-processed lactose, D-glucose, and D-galactose.

2. FD&C Blue No. 2 is very unstable, particularly when exposed to light, and the sugars significantly accelerate the decomposition of this

colorant. The decomposition of FD&C Blue No. 2 appears to proceed by reduction to a semiquinone followed by oxidation.

3. There appears to be some evidence to indicate molecular changes in the lactose upon storage at high temperature. These changes seem to interfere with the spectra analysis in the ultraviolet range for colorants which absorb between 228 and 284 mμ.

As a result of this investigation, further studies are underway to thoroughly evaluate the thermal stability of lactose and lactose-and-colorants, both in aqueous solutions and in solid dosage forms. In addition studies are continuing to elucidate the degradation reaction for FD&C Blue No. 2.

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Drug Standards

Assay of Progesterone in Oil Injectables

By JACOB WOLFF

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THE U.S.P. XVI (1) assay procedure for progesterone injection is a welcome simplification of the method in the previous edition (2). However, the assay is still based on the reaction with 2,4-dinitrophenylhydrazine, a general reagent for aldehydes and ketones. The melting point of the resulting hydrazone is rather high, 267–275°, and no other purity criteria are specified. Monty (3) reports that some chromatographically inhomogeneous 2,4-dinitrophenylhydrazones exhibit melting points no lower than the accepted values for the pure compounds. Umberger (4) used the color reaction with isonicotinic acid hydrazide for the determination of progesterone and testosterone. This reaction is more specific since it depends on the presence of a conjugated carbonyl linkage as in Δ^4-3-ketosteroids. Touchstone and Murawec (5) used spectrofluorometric techniques for the determination of progesterone in biological fluids. It appeared that a procedure based on the physical separation of progesterone from oil solutions, with its subsequent assay and identification, would be a more desirable approach than any of these.

Column chromatographic techniques have been successfully applied to similar problems.

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colorant. The decomposition of FD&C Blue No. 2 appears to proceed by reduction to a semiquinone followed by oxidation.

3. There appears to be some evidence to indicate molecular changes in the lactose upon storage at high temperature. These changes seem to interfere with the spectra analysis in the ultraviolet range for colorants which absorb between 228 and 284 mμ.

As a result of this investigation, further studies are underway to thoroughly evaluate the thermal stability of lactose and lactose-and-colorants, both in aqueous solutions and in solid dosage forms. In addition studies are continuing to elucidate the degradation reaction for FD&C Blue No. 2.

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Drug Standards

Assay of Progesterone in Oil Injectables

By JACOB WOLFF

A method is presented for the quantitative determination of progesterone in oil solutions. The ketosteroid is isolated by partition chromatography using nitromethane on purified siliceous earth as the immobile phase and *n*-heptane as the eluent. The assay is based on the ultraviolet absorbance in ethanol solution at the maximum near 241 mμ. The identity of the separated progesterone is confirmed by determining the infrared spectrum in carbon disulfide solution.

THE U.S.P. XVI (1) assay procedure for progesterone injection is a welcome simplification of the method in the previous edition (2). However, the assay is still based on the reaction with 2,4-dinitrophenylhydrazine, a general reagent for aldehydes and ketones. The melting point of the resulting hydrazone is rather high, 267–275°, and no other purity criteria are specified. Monty (3) reports that some chromatographically inhomogeneous 2,4-dinitrophenylhydrazones exhibit melting points no lower than the accepted values for the pure compounds. Umberger (4) used the color reaction with isonicotinic acid hydrazide for the determination of progesterone and testosterone. This reaction is more specific since it depends on the presence of a conjugated carbonyl linkage as in Δ^4-3-ketosteroids. Touchstone and Murawec (5) used spectrofluorometric techniques for the determination of progesterone in biological fluids. It appeared that a procedure based on the physical separation of progesterone from oil solutions, with its subsequent assay and identification, would be a more desirable approach than any of these.

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Carol (6) separated progesterone and testosterone using alcohol on purified siliceous earth as the immobile phase and iso-octane as the mobile solvent. Wotherspoon and Bedoukian (7) determined oxygenated compounds in essential oils by stripping from silica gel with ether after eluting the oils with pentane. Pesticides may be separated from fats and oils by the acetonitrile-hydrocarbon partition of Jones and Riddick (8) followed by chromatography on Florisil,[1] magnesium oxide, or purified siliceous earth. Umberger (4) obtained separation of progesterone or testosterone from oil, adequate for his colorimetric assay procedure, using Florisil and eluting with 2:1 iso-octane-chloroform. Tappi and co-workers (9) separated a number of steroidal hormones from each other and from olive oil using Florisil with successively more polar solvent mixtures. Snair and Schwinghamer (10) obtained a partial separation of estradiol dipropionate from oil using a column of siliceous earth and iso-octane thickened with polyethylene glycol 600, hexane being used as the mobile solvent. Monty (3) separated the 2,4-dinitrophenylhydrazones of aliphatic aldehydes and ketones on a nitromethane-Kieselguhr column with petroleum ether as the mobile solvent.

The proposed method was chosen after rather extensive experimentation with immiscible solvent systems and adsorbants. While many other techniques separated the main portion of the oil in the control samples, there was a steroid-like fraction which tended to remain with the progesterone throughout the various manipulations. Only the proposed procedure reduced the background absorbance to negligible proportions.

PROCEDURE

Materials.—Infrared spectrophotometer, a double beam Perkin-Elmer model 21; adsorbant, purified siliceous earth,[2] immobile solvent, nitromethane C.P.;[3] eluent, redistilled *n*-heptane; chromatographic column.

Insert a plug of purified cotton at the constriction of a chromatographic tube about 200 mm. long and about 25 mm. in internal diameter. Mix 8.0 ml. of nitromethane with 7.0 Gm. of adsorbant in a 150-ml. beaker until uniformly moistened. Transfer to the chromatographic tube in three approximately equal portions, tamping down with a packing rod after each addition. Arrange a long-stemmed, glass-stoppered, 250-ml. separator to serve as a reservoir for maintaining a constant level of liquid in the tube.

Standard Preparation.—Dissolve in alcohol 20.0 mg. of U.S.P. Progesterone Reference Standard, previously dried in vacuum over silica gel for 4 hours, dilute with ethanol to exactly 100 ml., and mix. Dilute 5.0 ml. of the solution with ethanol to make exactly 100 ml. and mix.

Assay Preparation.—Transfer, by means of accurately calibrated hypodermic syringe, 1.00 ml. of oil solution to a suitable volumetric flask so that the final solution will contain about 1 mg. of progesterone per ml. Dissolve in redistilled *n*-heptane, make to volume, and mix.

Assay.—Transfer 4.0 ml. of the assay preparation to the prepared chromatographic column. Pass 135 ml. of redistilled *n*-heptane through the column and discard the eluate.[4] Place a 200-ml. volumetric flask under the column as a receiver, add an additional 175 ml. of *n*-heptane to the column, and collect the entire eluate. Fill to the mark with *n*-heptane, and mix.

Transfer a 50-ml. aliquot to a 150-ml. beaker and save the remainder for the Identification Test. Evaporate to dryness under a gentle current of air on a steam bath. Remove the last traces of heptane by adding 1 ml. of methanol and redrying. Dissolve the progesterone by warming a few minutes in about 10 ml. of ethanol. Cool and dilute to exactly 100 ml. with ethanol.

Determine the absorbances of the sample and standard solutions relative to an alcohol blank at the peak near 241 mμ and at 300 mμ in a suitable spectrophotometer using matched 1-cm. cells. Calculate the quantity of progesterone in mg. in the aliquot taken from the formula $4\ A_u/A_s$, where A_u is the absorbance of the sample at 241 mμ minus the absorbance of the sample at 300 mμ, and A_s is the corresponding value for the standard.

Identification Test.—Evaporate the remainder of the eluate to dryness as discussed, starting with a 400-ml. beaker and transferring to a 5- or 10-ml. glass-stopper Erlenmeyer flask for the final evaporation. Dissolve the residue in 1.0 ml. of carbon disulfide, and determine the infrared absorption spectrum between 2 and 15 μ in a 1-mm. sodium chloride cell, using carbon disulfide in a matched cell as the blank. The resulting spectrogram should show all the significant absorption bands—and no others—that are found on a spectrogram similarly obtained with 3 mg. of U.S.P. Progesterone Reference Standard in 1.0 ml. of carbon disulfide.

DISCUSSION

In most chromatographic procedures, 1 ml. or less of immobile solvent is used per gram of adsorbant.

TABLE I.—ANALYSIS OF SYNTHETIC SOLUTIONS

Progesterone, mg./ml.	Adjuncts	% Recovered	
10.00	None	98.6	100.8
25.11	None	100.0	101.0
99.96	8% Benzyl alcohol 2% Ethanol	98.2	98.2

[1] Marketed by Floridin Co., Tallahassee, Fla.
[2] Acid-washed Celite 545, Johns-Manville Corp.
[3] Certified reagent grade from Fisher Scientific Co. was found to be satisfactory without further purification.

[4] The optimal volume of fore-run may vary by about 10 ml. with different batches of *n*-heptane or nitromethane. When new bottles of these reagents are used, determine the proper fore-run by the following procedure: Collect the last 25 ml. of the fore-run separately, and collect a tail cut of 25 ml. by passing through more eluant after collecting the main fraction in the 200-ml. flask. Evaporate these fractions as in the assay and make up to 25 ml. with ethanol. Determine the absorbance at 220, 241, and 300 mμ. An absorbance at 241 mμ exceeding that at 220 mμ indicates the presence of progesterone in the fraction involved, and the volume of the fore-run should be adjusted accordingly.

TABLE II.—ANALYSIS OF COMMERCIAL PROGESTERONE INJECTABLES

Sample	Declared mg./ml.	Adjuncts	Suggested Procedure % of Declared	Suggested Procedure Average	U.S.P. Assay % of Declared
1	100	8% Benzyl alcohol	86.2		
		2% Ethanol	87.0		
			86.8		
			87.6	86.9	90.6[a]
2	25	0.5% Chlorobutanol	95.0		
			93.8	94.4	96.8
3	10	0.1% Propylparaben	85.8		
			86.6	86.2	81.1
4	25	0.1% Propylparaben, in vegetable	80.3		
		oil	80.9	80.6	83.5
5	50	0.1% Propylparaben	86.5		
		20% Benzylbenzoate	87.5	87.0	88.9
6	25	0.5% Chlorobutanol	94.2		
			92.9		
			92.0	93.0	91.3
7	25	1:4000 Benzethonium octylacetate,	75.8		
		in peanut oil	75.4	75.6	75.3

[a] Determined m.p. at 260–264° (267–275° in U.S.P. XVI).

Here it was found that a relative excess of immobile solvent gave more consistent results. Evaporation to dryness and redissolving in alcohol are necessary to remove not only the heptane, but also the nitromethane which absorbs strongly in the ultraviolet region.

The progesterone recovered from oil injectables is usually slightly contaminated with ultraviolet absorbing substances. Consequently, the absorption spectra of samples and standards of equal concentration do not coincide exactly. While this difference may be appreciable at 220 mμ, it was never greater than 2% at 241 mμ. Prewashing the column with heptane did not reduce the background. Equilibrating the mobile and immobile solvents with each other increased the background, possibly by increasing the amount of nitromethane in the eluate. The interference, in any case, does not seem to affect the assay results. The absorbance of progesterone at 300 mμ is very low. On the samples, however, it ranged from 1–5% of the absorbance at the maximum around 241 mμ. It was found empirically on the samples of known concentration that subtracting this absorbance from that at the peak gave better results.

RESULTS

The recovery of progesterone from oil solutions was determined on solutions of known concentration. Weighed samples of U.S.P. Progesterone Reference Standard were dissolved by warming in 1 ml. of ethanol. About 4 ml. of sesame oil was added and the ethanol removed by heating on a steam bath for several hours. The solution was cooled and diluted to the desired volume. To check the reliability of the procedure under adverse conditions, the sesame oil used was from an old, somewhat oxidized sample. Duplicate determinations on three such samples are given in Table I and indicate that the recoveries are accurate within a 2% range.

In Table II, the results of the analysis of seven commercial preparations by the proposed method are compared with the results by the U.S.P. XVI (1) procedure. Except for samples 1 and 3, the results of the two procedures are in fair agreement. In sample 1, the difference may be explained in part by the impurities present in the hydrazone obtained in the U.S.P. procedure as indicated by the low melting point. Only two of the seven samples, 2 and 6, meet the requirements of the U.S.P. by either method of analysis.

The infrared absorption spectra of the progesterone recovered in the samples examined were indistinguishable from those of the reference standard.

SUMMARY

A method has been proposed for the assay of progesterone in oil solution, based on the isolation of the active ingredient by partition chromatography. The method is more specific than the current U.S.P. method and yields comparable results.

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Synthesis of Pyridine-2-aldoxime-C^{14} Methiodide (2-PAM-C^{14} Iodide)

By LEON CLARK and LLOYD J. ROTH

A procedure is described for the synthesis of C-14 oxime-labeled pyridine-2-aldoxime methiodide. Picolinamide-C^{14} is prepared from 2-bromo-pyridine and potassium cyanide-C^{14}. This amide is used in the synthesis of 2-picolinyl-C^{14}-benzenesulfonyl hydrazide which, upon decomposition with heat and alkali, yields pyridine-2-aldehyde-C^{14}. Pyridine-2-aldoxime-C^{14} is readily obtained from the aldehyde. Treatment of the oxime with methyl iodide in benzyl alcohol yields the desired 2-PAM iodide.

INTEREST in 1-methyl-2-pyridinium aldoxime iodide or pyridine-2-aldoxime methiodide (2-PAM iodide) is largely a result of its effectiveness as an antidote for organic phosphate poisoning. The ever-increasing use of highly toxic organophosphorus compounds as agricultural insecticides as well as potential military nerve gases makes it a drug of importance. In addition, 2-PAM iodide has found important research application in the study of enzyme systems involving cholinesterase inhibitors.

2-PAM iodide labeled with C-14 makes possible the study of this drug and/or its metabolites within brain and other body tissues. Studies utilizing oxime-labeled as well as methyl-labeled PAM iodide are desirable. The lack of any published procedure for the synthesis of oxime-labeled 2-PAM, and the potential usefulness of this compound as a research tool, led us to develop the synthesis herein reported.

EXPERIMENTAL

Picolinamide-C^{14}.—Two major considerations entered into the choice of this compound as a starting point in the synthesis of C^{14}-labeled pyridine-2-aldehyde. First, because it can be prepared from cyanide-C^{14}, it affords a convenient means for the introduction of a labeled atom. Secondly, the amide can be used directly in the synthesis of the required hydrazide thus circumventing the problem of esterification of picolinic acid.

The preparation of picolinamide from 2-bromopyridine and potassium cyanide has been reported by Brode and Bremer (1). Inasmuch as these workers were concerned with optimum yield based upon bromopyridine, the procedure had to be redesigned with a view toward the achievement of optimum yield based upon cyanide.

A solution of potassium cyanide-C^{14} (390 mg., 6m*M*, 10 mc.) in water (1 ml.) was prepared in a heavy-walled Pyrex reaction tube. The tube and contents were kept cold by means of an ice water bath as cuprous chloride (250 mg.), added in small portions, was brought into solution with shaking and swirling. To the solution thus prepared were added, in order, 790 mg. (5 m*M*) of 2-bromopyridine dissolved in 1 ml. of ethanol, and 100 mg. of potassium carbonate. The tube was then sealed and heated for 40 hours at 180 to 200°.

The tube was allowed to cool to room temperature, chilled in an ice bath, then opened and the contents evaporated to dryness under reduced pressure. The residue was redissolved in hot dilute ammonium hydroxide. This ammoniacal solution was then made slightly acidic with hydrochloric acid and treated with hydrogen sulfide. Copper sulfide was filtered off, and the filtrate evaporated to dryness *in vacuo*. The residue was dissolved in a minimum volume of water, neutralized with potassium carbonate, and submitted to continuous extraction with ether for 2 hours. Crude picolinamide-C^{14} was obtained upon evaporation of the solvent. The product was purified by sublimation *in vacuo*, m.p. 105–106° [reported m.p., 106.3 to 106.8° (1)]. Yield: 182 mg. (25%), based on cyanide.

During the course of preliminary research with unlabeled materials, the product was further characterized by hydrolysis to the corresponding acid, isolated as the copper salt. The acid obtained by regeneration from the copper salt was chemically indistinguishable from an authentic purified specimen of picolinic acid.

Picolinic-C^{14} Acid Hydrazide.—The amide from the previous step was dissolved in absolute alcohol (5 ml.) and treated with 0.5 ml. of hydrazine hydrate (99–100%). This solution was heated under reflux for 48 hours on a steam bath. Solvent was then removed under reduced pressure. The product was purified by recrystallization from benzene. Yield: 169 mg. (82%), m.p. 98–99° [reported m.p. 100° (2)].

2-Picolinyl-C^{14}-benzenesulfonylhydrazide.—The above product in pyridine solution was treated with benzenesulfonyl chloride in accordance with the method of Niemann, Lewis, and Hays (3). The resultant benzenesulfonylhydrazide was purified by recrystallization from boiling alcohol. Yield: 230 mg. (68%), m.p. 202–203° [reported m.p. 202–203.5° (3)].

Pyridine-2-aldoxime-C^{14}.—Use was made of the reaction of McFadyen and Stevens (4) in the production of pyridine-2-aldehyde-C^{14} from the above benzenesulfonylhydrazide. Inasmuch as this reaction imposes rather severe conditions of temperature and alkalinity upon the relatively unstable aldehyde, it was deemed imperative that the

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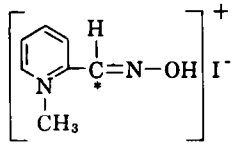
product be isolated from the reaction mixture as rapidly as possible. Accordingly, the reaction was carried out under vacuum, using an all-glass apparatus with appropriate ground glass, Apiezon (N) lubricated fittings. In essence, this apparatus consisted of a vacuum trap with a separable receiver and a side arm to accommodate a ground glass-jointed reaction tube.

With a mixture of the above benzenesulfonylhydrazide, anhydrous sodium carbonate (200 mg.) and glycerol (2 ml.) in the reaction tube, the system was evacuated to 10^{-5} mm. of Hg. The lower portion of the receiver-trap was surrounded by a liquid nitrogen bath while the reaction tube was gently heated with a micro burner. The reaction proceeded with a vigorous evolution of gas. The condensable fraction of the gas collected as an ice in the cold trap. The noncondensable portion was pumped into the exhaust system of an efficient hood which was equipped with a high-efficiency filter.[1]

When the evolution of gas had practically ceased, the evacuated trap was closed off and permitted to warm to room temperature. The separable receiver (a 1 × 10 in. test tube with ⅋ 24/40 outer joint) was used as a reaction vessel in the next operation.

To the water-clear liquid in the receiver tube was added hydroxylamine hydrochloride (20 mg.), sodium bicarbonate (25 mg.), and water (1 ml.). The resultant mixture was heated for 10 minutes on a steam bath. The oxime was isolated by continuous extraction with ether and purified by recrystallization from benzene-petroleum ether. Yield 20.2 mg (20%), m.p. 112–113° [reported m.p. 113.5° (5)].

Radiochemical purity of the pyridine-2-aldoxime-C^{14} was determined by means of paper chromatography, using a water-saturated benzene system and a water-saturated chloroform system. In each instance, an autoradiogram of the chromatographic strip showed a single band of radioactivity.



Pyridine-2-aldoxime-C^{14} Methiodide

Pyridine-2-aldoxime-C^{14} Methiodide.—Whereas pyridine-2-aldoxime methiodide has been prepared by heating the oxime with methyl iodide in a suitable solvent (6), this procedure proved unsatisfactory when applied to production of the radiochemically pure compound on a millimole scale. Thus, a scheme of synthesis was sought whereby side reactions could be minimized or eliminated by conducting the reaction at a lower temperature. The use of benzyl alcohol as a catalytic solvent (7) proved a satisfactory solution to the problem.

The oxime from the previous step was thoroughly mixed with 0.25 ml. of freshly distilled benzyl alcohol in a glass-stoppered test tube. To it was added 0.25 ml. of freshly distilled methyl iodide. The tube was securely stoppered and set aside for 1 week at room temperature. An additional 0.25-ml. portion of methyl iodide was then added and the mixture set aside for 4 or 5 days. In the meantime, bright yellow crystals of the methiodide had formed in the reaction mixture. This mixture was added, dropwise with stirring, to 100 ml. of anhydrous ether. The precipitate was collected on a sintered-glass filter, washed thoroughly with anhydrous ether, and dried in air. A sample for microanalysis was dried for several days *in vacuo*, at room temperature. Yield: 39.3 mg. (90%); m.p. 225–226° (decompn.) [reported m.p. 225–226° (decompn.) (8)].

Anal.—Calcd. for $C_7H_9IN_2O$: C, 31.8; H, 3.41; N, 10.6; I, 48.1. Found: C, 32.1; H, 3.45; I, 47.96; N, 10.5. I, 47.96.

Radiochemical purity of the pyridine-2-aldoxime-C^{14} methiodide was determined by means of paper chromatography, using a *n*-butanol:acetic acid:water (5:1:3) system, and a water-saturated *n*-butanol system. In each case an autoradiogram of the chromatographic strip showed a single band of radioactivity. Radiochemical yield 0.232 millicuries (2.3% based on potassium cyanide).

SUMMARY

1. Pyridine-2-aldoxime-C^{14} has been synthesized and used in the preparation of the corresponding methiodide.

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[1] This filter is rated as 99.98% efficient for removal of particles in the order of 0.4 μ in diameter and manufactured by Flanders Filters, Inc., Riverhead, N. Y.

Relationship of *in Vitro* Release to Urinary Recovery in Man of a Sustained-Release Preparation of S^{35} Prochlorperazine

By EARL ROSEN

A sustained-release preparation of S^{35}-labeled prochlorperazine prepared with different *in vitro* release patterns as determined by the rotating bottle method was administered to human subjects. Urinary recovery of S^{35} was determined by liquid scintillation counting. Previously published data on sustained-release preparations of phenylpropanolamine and trimeprazine, treated in the same fashion as the prochlorperazine data, are included. For the specific dosage form of each drug, a direct rank relationship was found to exist between *in vitro* data and the respective *in vivo* data.

EARLY IN the development of sustained-release products it was recognized that a laboratory test method could not be expected to duplicate the *in vivo* release of a product, but should provide an artificial means of assuring batch-to-batch reproducibility of a product proved to be clinically effective on the basis of controlled studies in humans (1, 2). Though numerous sustained-release products have been developed in the past, little objective human data have been reported which correlates differences in *in vitro* release patterns with *in vivo* response. Or, to put it another way, few data have been published to directly correlate the *in vitro* release pattern with clinically satisfactory material of a specific formulation.

This study was undertaken to establish that, for various dosage regimens of sustained-release formulations of S^{35}-labeled prochlorperazine, a relationship exists between *in vitro* release by the Souder and Ellenbogen technique (2) and human *in vivo* response. In addition, the experiment was designed to provide data to verify that the *in vitro* release limits used for *in vitro* control of lot-to-lot reproducibility are realistic.

The procedure utilized in this study is one previously described (3) for preparing radiochemically labeled formulations of pelleted sustained-release medication. The basic approach involves the preparation of "fast," "normal," and "slow" releasing S^{35}-labeled prochlorperazine formulations and the measurement of the radioactivity appearing in the urine after administration to human subjects.

It was recognized that this plan involved certain assumptions, namely, that the human body metabolizes prochlorperazine from any of the three formulations in the same manner, and, secondly, that the observed radioactivity measures only the phenothiazine moiety. In order to validate the treatment of experimental data and to support the conclusions, previously published data by Heimlich, *et al.* (4, 5), were subjected to a method of analysis permitting direct comparison of *in vitro* and *in vivo* results.

EXPERIMENTAL

***Preparation* of Dosage Forms.**—Chemical and radiochemical purity of S^{35} prochlorperazine dimaleate were established by spectrophotometric analysis, infrared analysis, chloride determination, half-life determination, and chromatographic analysis. The labeled compound was synthesized with a specific activity requiring no dilution with unlabeled chemical prior to formulation.

The general procedure used for preparation of sustained-release pellets was the same as previously reported by Rosen and Swintosky (3). The various sustained-release groups thus prepared were combined in varying proportions into three different pellet mixtures each of which had a different *in vitro* release pattern. Each mixture was formulated as capsules containing 15 mg. of prochlorperazine as the dimaleate salt, with an activity of 3 μc./mg. at the time of administration.

The *in vitro* release patterns of prochlorperazine were determined using the apparatus and general method reported by Souder and Ellenbogen (2). The percentage of drug released at specified intervals was obtained by difference after spectro-

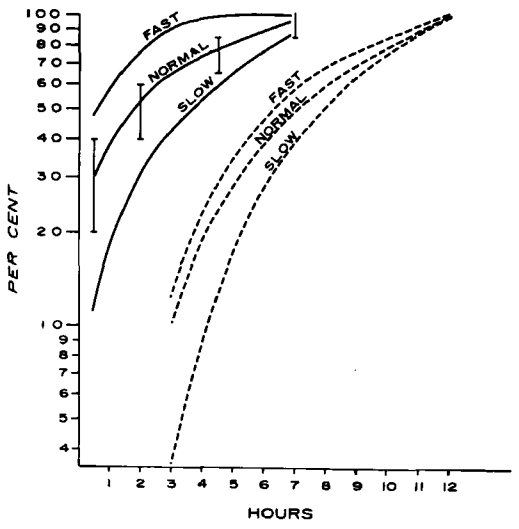

Fig. 1.—Comparison of *in vitro* release and *in vivo* measurements. Solid lines ——— represent per cent *in vitro* release of "slow," "normal," and "fast" pellet type S^{35} prochlorperazine sustained-release formulations. Bars indicate control limits for a specific pellet-type product. Broken line------ represents per cent average cumulative 12-hr. urinary recovery from adult human subjects after administration of "slow," "normal," and "fast" formulations.

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photometrically determining the amount of drug in the undisintegrated residual pellets at each test interval. The *in vitro* release patterns of the three S^{35}-labeled prochlorperazine formulations are given in Fig. 1.

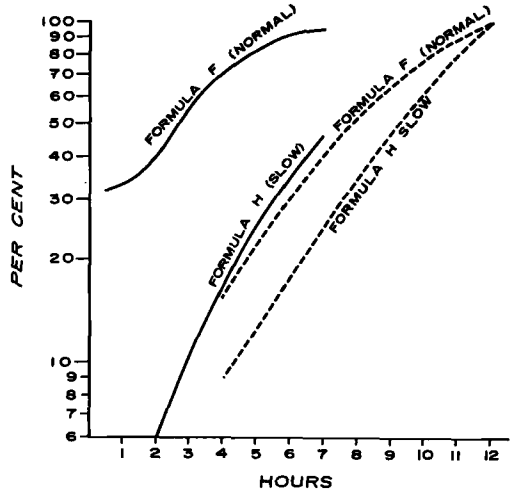

Fig. 2.—Comparison of *in vitro* release and *in vivo* measurements. Solids lines ——— represent per cent *in vitro* release of Formula "F" ("normal") and Formula "H" ("slow") pellet-type trimeprazine sustained-release formulations (4). Broken lines - - - - - - represent per cent average cumulative 12-hr. urinary recovery from adult human subjects after administration of Formula "F" ("normal") and Formula "H" ("slow") (4).

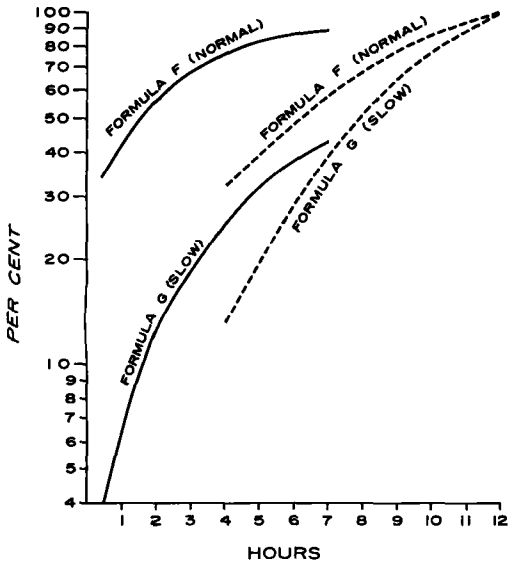

Fig. 3.—Comparison of *in vitro* release and *in vivo* measurements. Solid lines ——— represent per cent *in vitro* release of Formula "F" ("normal") and Formula "G" ("slow") pellet-type phenylpropanolamine sustained-release formulations (5). Broken lines - - - - - - represent per cent average cumulative 12-hr. urinary recovery from adult human subjects after administration of Formula "F" ("normal") and Formula "G" ("slow") (5).

Analysis of Specimens.—Urine samples were counted in a liquid scintillation counting solution adapted from one devised by Kinard (6). This solution consists of dioxane, 400 ml.; naphthalene, 80 Gm.; PPO,[1] 5 Gm.; and POPOP,[2] 50 mg. A sample of urine (0.4 ml. or less) is added to 10 ml. of liquid phosphor contained in a sample vial and, after mixing, this solution is counted in a model 314 Tri-Carb[3] liquid scintillation spectrometer. An internal standard is then added, the sample is again counted and, after proper corrections are made for quench, the counting rate of the urine sample is obtained.

Decay corrections were employed to permit a direct comparison of all experimental results.

Human Studies.—A total of 16 healthy adult subjects were given one or more of the sustained-release formulations. Of these, seven received a second formulation after a lapse of 2 weeks. Seven individuals received the "fast" formulation, nine the "normal" formulation, and seven the "slow" formulation, for a total of 23 observations. All subjects were given the drug in the morning, and urine samples were collected in 3-hr. increments over a 12-hr. period.

RESULTS AND DISCUSSION

In order to provide a basis for direct comparisons of the experimental *in vitro* and *in vivo* data, the per cent of the cumulative 12-hr. S^{35} to be excreted (Fig. 1) was plotted. The *in vitro* and *in vivo* data of Heimlich, *et al.*, on "normal" and "slow" formulations of trimeprazine (4) and "normal" and "slow" formulations of phenylpropanolamine (5) are treated in the same manner in Fig. 3.

Inspection of Figs. 1, 2, and 3 shows a direct rank relationship of the *in vivo* and *in vitro* data, indicating that "slow" or "fast" *in vitro* release patterns correlate with respective *in vivo* measurements. The data also show that formulations outside of the *in vitro* control limits can be differentiated *in vivo*.

For the formulations studied, differences in release are more demonstrable by the *in vitro* test than by *in vivo* response. This feature of the rotating bottle technique provides a measure of assurance that in the cited instances the test is sufficiently sensitive to discriminate between materials which might and those which might not produce a satisfactory clinical effect.

In vitro control specifications of 20–40% at 0.5 hour, 40–60% at 2 hours, 65–85% at 4.5 hours, and not less than 85% at 7 hours are rational and meaningful for control of this particular sustained-release prochlorperazine formulation.

It is apparent from this study that when biochemical measurements of a drug and its metabolites in humans are available in conjunction with objective and subjective clinical studies, there exists a scientific basis for evaluating the effectiveness of sustained-release formulations and for establishment of a realistic *in vitro* procedure to assure lot-to-lot reproducibility of the specific formulation prepared in the specific manner.

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[1] 2,5-Diphenyloxazole.
[2] 1,4-Bis-2[5-phenyloxazolyl]-benzene.
[3] Packard Instrument Company, Inc., LaGrange, Ill.

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(4) Heimlich, K. R., MacDonnell, D. R., Polk, A., and Flanagan, T. L., THIS JOURNAL, 50, 213(1961).
(5) Heimlich, K. R., MacDonnell, D. R., Flanagan, T. L., and O'Brien, P. D., *ibid.*, 50, 232(1961).
(6) Kinard, F. E., *Rev. Sci. Instr.*, 28, 293(1957).

X-ray and Crystallographic Applications in Pharmaceutical Research III. Crystal Habit Quantitation

By JOHN W. SHELL†

It is customary practice when describing the crystal habit of a given compound to make use of qualitative terms, such as "needles" or "plates." These terms are useful in describing crystal habit as it relates to some important pharmaceutical characteristics, such as suspension stability and syringeability, but inadequate for the more involved processes in which crystal habit affects tableting ability. In this paper a method is presented for describing the crystal habit of a given compound in quantitative terms which may be used, in some instances, to predict tableting behavior and to serve as specifications for tableting materials.

THE SYMMETRY of a crystal is fixed by the crystal system and class to which it belongs. Its relative dimensions, however, are independent of its symmetry. As a crystal grows from solution, a variety of factors, notably crystallization rate and the presence of impurities, tend to influence the amount of growth on each of the possible faces. Extremes of the possible conditions result in acicular, or needle-shaped crystals as a consequence of unidimensional growth (bidimensional retardation) and tabular, or plate-shaped crystals, as a consequence of bidimensional growth (unidimensional retardation). Terms such as acicular, equant, and tabular describe crystal habit in a qualitative manner.

Crystal habit often exerts a dominant influence on some important pharmaceutical characteristics, such as suspension stability, suspension syringeability, and the behavior of powder mixes during a tablet-compressing process. In the case of suspension syringeability, the influence is mostly mechanical. A suspension of plate-shaped crystals, for instance, may be injected through a small needle with greater ease than one with needle-shaped crystals of the same overall dimensions.

In the case of tableting behavior, however, the influence of the crystal habit of the active ingredient is more involved. The mechanical influence of crystal shape just mentioned is one factor, but there is another, sometimes dominant one, which results from the anisotropy of cohesion and of hardness which is possessed by organic (low symmetry) crystals and, therefore, of most pharmaceutically important compounds. It is significant that this anisotropy bears a fixed relation to the fundamental crystallographic directions. Therefore, as crystal habit varies, the dominant faces may vary in their relation to this anisotropy, and it is the influence of the dominant faces which tends to orient the crystals during a packing or compression process. Thus, major habit variations of an active ingredient can influence greatly the ease or the difficulty of making satisfactory compressed tablets. This is particularly true when the active ingredient makes up a large portion of the total tablet mass.

In order to evaluate tableting behavior as influenced by crystal habit, the habit must be expressed in quantitative terms which reflect some relationship between the dominant faces and the principal crystallographic directions. Qualitative terms describing shape are, in some instances, not sufficient.

Relating the Dominant Faces to the Crystallographic Directions.—An ideal situation exists when the crystals are less than about 0.2 μ in size, for in this range there is measurable "line-broadening" in the X-ray powder diffraction pattern, and the average crystal sizes in each of the crystallographic directions can be measured directly. Thus, needle-shaped crystals elongated along the c axis show sharp (001) reflections, and broadened (hk0) reflections. The actual dimensions can be measured from the width of each of the appropriate peaks at half maximum height. However, most crystalline preparations for pharmaceutical use are out of the X-ray line-broadening range, being larger, usually, than one micron, and this procedure cannot be applied.

For a typical pharmaceutical composition, it has been found that a quantitative description of crystal habit as it affects tableting behavior can be based upon measurements of preferred orientation. After relating habit extremes to tableting behavior by experimentation, optical and X-ray crystallographic studies on representative single crystals allow the designation of the dominant faces by their Miller indexes. An X-ray powder diffraction

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For a typical pharmaceutical composition, it has been found that a quantitative description of crystal habit as it affects tableting behavior can be based upon measurements of preferred orientation. After relating habit extremes to tableting behavior by experimentation, optical and X-ray crystallographic studies on representative single crystals allow the designation of the dominant faces by their Miller indexes. An X-ray powder diffraction

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pattern is then measured for crystals of each habit extreme on specially prepared samples. The samples are prepared in such a manner that preferred orientation effects are maximized. A ratio of the relative peak intensities of critical lines in this diffraction pattern serves to indicate the average habit of the crystals. The ratio is useful in predicting tableting behavior, as well as serving, when desired, as a manufacturing specification.

General Illustrative Example.—In the preparation of compressed tablets of a specific drug, it has been found that some lots of bulk material vary in their ability to compress readily into satisfactory tablets. Although the pure crystalline drug in the various lots displayed no variation in measurable physical constants, examination with a petrographic microscope has disclosed a crystal habit variation. Crystals from lots with histories of good tableting behavior exhibited an acute bisectrix interference figure as the most common orientation, and crystals from lots with bad tableting behavior histories exhibited obtuse bisectrix figures most commonly. A consideration of the optic orientation (a = Y, b = X) and optic sign (positive) of single crystals indicated that the c crystallographic axis was coincident with the acute bisectrix direction, and the b axis with the obtuse bisectrix direction. Thus, it followed that "poor lot" crystals tended to lie on their 010 face, and "good lot" crystals tended to lie on their 001 face. Figure 1 shows orthogonal and clinographic projections of the crystal. The projections do not show the 001 face, although this face sometimes is present from growth, and often from cleavage, resulting in crystals with favorable tableting ability.

Using single crystal X-ray data,[1] an X-ray powder diffraction pattern was partially indexed. This allowed the choosing of powder pattern peaks whose intensities would most likely be influenced by crystals changing from 001 orientation to 010 orientation. These peaks were found in the diffraction pattern at 2 θ values of 8.72° (intensity indicates 010 orientation) and 12.09° (intensity indicates close to 001 orientation). (The 001 reflection itself is extinct.[2])

Samples for analysis were prepared so that all crystals had the best chance to orient preferentially on their most prominent faces. To accomplish this the sample was dusted onto a petrolatum-smeared glass microscope slide, and the excess shaken off. The essentially monolayered sample was placed directly in a diffractometer (General Electric XRD-5) and the intensities of the peaks at 8.72 and 12.09° were determined from the recorder tracing.

TABLE I.—CORRELATION OF TABLETING ABILITY OF VARIOUS LOTS OF BULK DRUG WITH CRYSTAL HABITS

Order of Tableting Ability of Lots		Intensity at 12.09°/Intensity at 8.72°
A		3.60
B		2.36
C		2.43
D	decreasing	1.50
E	tableting	2.00
F	ability	1.40
G		1.25
H		1.15
I	↓	1.12

[1] The orthorhombic unit cell dimensions are: a = 9.07 A; b = 20.14 A; c = 7.78 A.

[2] Corresponding d spacings for these 2 θ values are: 10.13 A for 8.72°; 7.31 A for 12.09°.

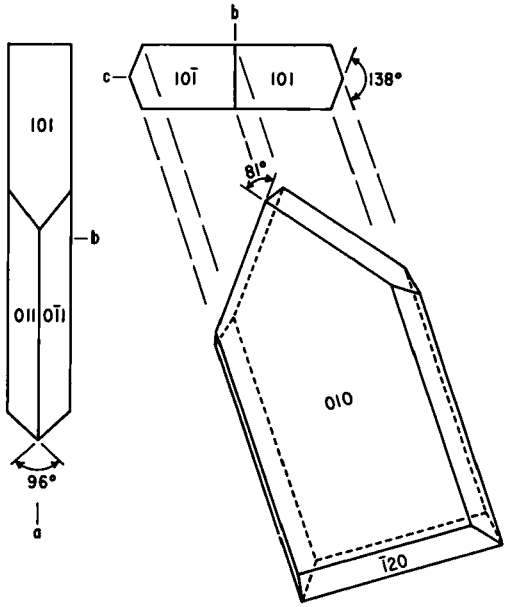


Fig. 1.—Orthogonal and clinographic crystal projections.

Since the intensity of the 12.09° peak is proportional to the mass of crystalline material with 001 orientation (good tableting ability) and that of the 8.72° peak proportional to the mass with 010 orientation (poor tableting ability), the ratio of intensities of these two peaks is a very sensitive measure of the average crystal habit represented, as it relates to tableting ability.

Table I shows a comparison of nine sample lots of bulk drug. In the left-hand column the designations are listed in decreasing order of acceptability, as determined by actual observation during the tableting process by experienced personnel. In the right-hand column are listed the intensity ratios for each sample designation as determined by the method described above. Although the averaging of multiple samplings of each lot gives better correlation, the measured values of Table I, representing single determinations, show good correlation with observed tableting behavior and indicate the validity of the method. The results reported are from a blind study.

As the method employs the use of measured ratios, it is independent of the total amount of sample taken for each determination. The validity is increased, however, by taking as large a sample as is practical and, for this reason, it has been found advisable to employ large X-ray beam and detector slits in order to scan larger sample areas. The attendant loss of resolution when large slit values are employed is not important for these studies.

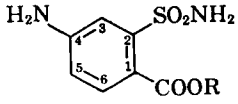
A total time of approximately 5 minutes is required for sample preparation, X-ray scanning, and ratio calculations.

Synthesis of Alkyl Esters of 4-Amino-2-sulfamoylbenzoic Acid

By GLENN H. HAMOR and MEHDI JANFAZA

Nine esters of 4-nitro-2-sulfamoylbenzoic acid were prepared by the alcoholysis reaction of passing hydrogen chloride into a refluxing solution of 6-nitrosaccharin in the appropriate alcohol. Reduction of these alkyl 4-nitro-2-sulfamoylbenzoates using hydrogen and palladium-on-carbon catalyst gave the following alkyl 4-amino-2-sulfamoylbenzoates: methyl; ethyl; *n*-propyl; isopropyl; *n*-butyl; *sec*-butyl; *n*-pentyl; 1-ethylpropyl; and *n*-hexyl. Also synthesized was isopropyl 6-amino-2-sulfamoylbenzoate. Many of these compounds possessed marked anticonvulsant activity as indicated by their prevention of the effect of strychnine or maximal electric shock in mice.

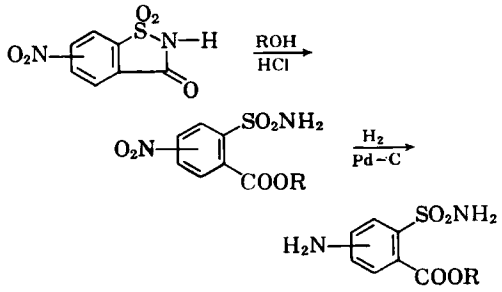
A NUMBER of alkyl esters of 4 (or 6)-amino-2-sulfamoylbenzoic acid were prepared for pharmacological screening. The ten compounds synthesized are listed and their properties given in Table I, and have the following formula



A

R = CH_3, C_2H_5, n-C_3H_7, i-C_3H_7, i-C_3H_7(6-NH_2), n-C_4H_9, s-C_4H_9, n-C_5H_{11}, $CH(C_2H_5)_2$, n-C_6H_{13}

Nine esters of 4-nitro-2-sulfamoylbenzoic acid (Table II) were prepared by the alcoholysis reaction of passing hydrogen chloride into a refluxing solution of 6-nitrosaccharin in the appropriate alcohol (1). The 6-nitrosaccharin was prepared by the permanganate oxidation of 4-nitro-2-sulfamoyltoluene (2). Isopropyl 6-nitro-2-sulfamoylbenzoate was synthesized by refluxing 4-nitrosaccharin in acidified 2-propanol. The 4-nitrosaccharin was prepared by nitration of 2-sulfobenzoic acid, followed by reaction with phosphorus pentachloride to form the diacid chloride, and then treatment with anhydrous ammonia (3). Reduction of these 4 (or 6)-nitro-2-sulfamoylbenzoates using hydrogen and palladium-on-carbon catalyst gave the desired amino esters. The reaction sequence is



Pharmacological testing has shown several of the alkyl 4-amino-2-sulfamoylbenzoates to possess marked anticonvulsant activity.[1] The most potent anticonvulsant compound in this series, as indicated by potency in preventing the effect of strychnine or maximal electric shock in mice, was the isopropyl ester. The methyl and ethyl esters were much less potent.[2]

EXPERIMENTAL

4-Nitro-2-sulfamoylbenzoic Acid Esters.—These esters were prepared by acidic alcoholysis of 6-nitrosaccharin (prepared according to Noyes) (2) in the appropriate alcohol. The compounds are listed and their properties given in Table II. The procedure used will be illustrated by the following account of the synthesis of ethyl 4-nitro-2-sulfamoylbenzoate. Two and one-half grams (0.01 mole) of 6-nitrosaccharin was dissolved in 25 ml. of absolute ethanol and poured into a dry 50-ml. two-neck flask. The solution was heated under reflux for 2 hours, with continuous passage of dry hydrogen chloride gas through the solution. The current of hydrogen chloride gas was discontinued and the reflux condenser removed; the solution was then heated for several minutes to expel hydrogen chloride. At the beginning of the reflux period it was noted that the color of the solution of 6-nitrosaccharin in ethanol was yellow, but as the reaction proceeded, it gradually became lighter, thus showing the progress of the reaction. On cooling and slow evaporation, the hot solution yielded crystals. Recrystallization from absolute ethanol gave 2.7 Gm. (90%) of crystalline solid, m.p. 139–141°.

Isopropyl 6 - Nitro - 2 - sulfamoylbenzoate.—Four and one-half grams (0.02 mole) of 4-nitrosaccharin (prepared according to Hamor) (3) was dissolved in 50 ml. of warm dry 2-propanol in a 100-ml. round-bottom flask fitted with a gas inlet tube and a reflux condenser topped by a calcium chloride drying tube. The solution was refluxed for 2 hours, with hydrogen chloride gas being passed in for 46 minutes of this time. Evaporation of the solvent and recrystallization of the resulting solid from 2-propanol, followed by washing with cold 2-propanol gave 2.8 Gm. (50%) of white crystalline compound, m.p. 130–133°.

4 - Amino - 2 - sulfamoylbenzoic Acid Esters.—These esters were prepared by reduction of the corresponding nitro compounds, using hydrogen and palladium-on-carbon catalyst. The compounds thus synthesized are listed and their properties given in Table I. The procedure used will be shown by the following account of the synthesis of ethyl

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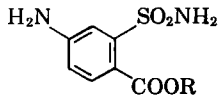
[1] The authors are indebted to Smith Kline and French Laboratories, Philadelphia, Pa., for pharmacological testing.

[2] Loev and Kormendy describe the synthesis of several of these compounds by a different method and give the preliminary pharmacological results in a recent paper (4).

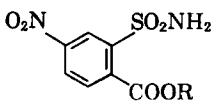
TABLE I.—ALKYL 4-AMINO-2-SULFAMOYLBENZOATES[a]



No.	R	Formula	M.p.,[b] °C.	Recrystallizing Solvent	Yield, %	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
1	CH_3	$C_8H_{10}N_2O_4S$	183[c]	Absol. ethanol	76	41.73	42.00	4.37	4.54
2	C_2H_5	$C_9H_{12}N_2O_4S$	152–153	Absol. ethanol	66	44.25	44.48	4.95	4.82
3	n-C_3H_7	$C_{10}H_{14}N_2O_4S$	148–149	Absol. ethanol	70	46.49	46.70	5.46	5.51
4	i-C_3H_7	$C_{10}H_{14}N_2O_4S$	120	Absol. ethanol	61	46.49	46.78	5.46	5.68
5	n-C_4H_9	$C_{11}H_{16}N_2O_4S$	108	Absol. ethanol	65	48.51	48.47	5.92	5.79
6	s-C_4H_9	$C_{11}H_{16}N_2O_4S$	102–104	Ethanol-H_2O	47	48.51	48.70	5.92	5.97
7	n-C_5H_{11}	$C_{12}H_{18}N_2O_4S$	93–94	Absol. ethanol	78	50.33	50.42	6.33	6.30
8	$CH(C_2H_5)_2$	$C_{12}H_{18}N_2O_4S$	121–123	Ethanol-H_2O	43	...	...	...	...
9	n-C_6H_{13}	$C_{13}H_{20}N_2O_4S$	103–104	Ethanol-H_2O	66	51.98	51.95	6.71	6.65
10	i-C_3H_7 (isopropyl 6-amino-2-sulfamoylbenzoate)	$C_{10}H_{14}N_2O_4S$	120.5–121.5	Ethanol-H_2O	58	46.49	46.70	5.46	5.58

[a] Analyses were performed by Elek Micro Analytical Laboratories, Los Angeles, Calif. [b] Melting points were taken either with a Fisher-Johns melting point apparatus or by the capillary tube method, and are uncorrected. [c] Reported m.p. 180° (5).

TABLE II.—ALKYL 4-NITRO-2-SULFAMOYLBENZOATES[a]



No.	R	Formula	M.p.,[b] °C.	Recrystallizing Solvent	Yield, %	Reaction Medium	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
11	CH_3	$C_8H_8N_2O_6S$	193[c]	Methanol	70	Methanol	36.92	37.01	3.09	3.08
12	C_2H_5	$C_9H_{10}N_2O_6S$	139–141	Absol. ethanol	90	Absol. ethanol	39.41	39.64	3.67	3.73
13	n-C_3H_7	$C_{10}H_{12}N_2O_6S$	102–103	1-Propanol	76	1-Propanol	41.66	41.81	4.19	4.19
14	i-C_3H_7	$C_{10}H_{12}N_2O_6S$	128	2-Propanol	52	2-Propanol	41.66	41.82	4.19	4.26
15	n-C_4H_9	$C_{11}H_{14}N_2O_6S$	87–88	1-Butanol	66	1-Butanol	43.70	43.91	4.66	4.69
16	s-C_4H_9	$C_{11}H_{14}N_2O_6S$	140–143	Ethanol-H_2O	60	2-Butanol	...	...	...	...
17	n-C_5H_{11}	$C_{12}H_{16}N_2O_6S$	77–79	Ethanol	50	1-Pentanol	45.56	45.45	5.09	4.98
18	$CH(C_2H_5)_2$	$C_{12}H_{16}N_2O_6S$	70–95	Ethanol-H_2O	33	3-Pentanol	...	...	...	...
19	n-C_6H_{13}	$C_{13}H_{18}N_2O_6S$	70–72	Ethanol	71	1-Hexanol	...	...	...	...
20	i-C_3H_7 (isopropyl 6-nitro-2-sulfamoylbenzoate)	$C_{10}H_{12}N_2O_6S$	130–133	...	49	2-Propanol	...	...	...	...

[a] Analyses were performed by Elek Micro Analytical Laboratories, Los Angeles, Calif. [b] Melting points were taken either with a Fisher-Johns melting point apparatus or by the capillary tube method, and are uncorrected. [c] Reported m.p. 191° (5).

4-amino-2-sulfamoylbenzoate. Ethyl 4-nitro-2-sulfamoylbenzaote (3.4 Gm., 0.012 mole) in 75 ml. of ethyl acetate was reduced with hydrogen at about 3 Atm. pressure with 1.5 Gm. of 5% palladium-on-carbon catalyst. Filtration of the mixture, using a small amount of a filter aid, and evaporation of the filtrate followed by recrystallization of the resultant solid from absolute ethanol gave 2.0 Gm. (66%) of ethyl 4-amino-2-sulfamoylbenzoate, m.p. 152–153°.

Isopropyl 6 - Amino - 2 - sulfamoylbenzoate.—This compound was synthesized by reduction of isopropyl 6-nitro-2-sulfamoylbenzoate in a manner similar to that used for preparation of the 4-amino-2-sulfamoylbenzoic acid esters. Its properties are listed in Table I.

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Formation of an Insoluble Condensation Product from Sulfamethizole and Formaldehyde

Sir:

Zinsser, *et al.* (1), recently reported on the composition of the sediment found in human urine after the ingestion of a combination of sulfamethizole, methenamine, and mandelic acid. Using the Bratton and Marshall assay procedure (2), more than 50% of the sediment was shown to be sulfonamide. In agreement with previous investigators (3), the sediment was also stated to contain ammonium salts and urates.

In vitro, we have investigated the compatibility of sulfamethizole, methenamine, and mandelic acid in the pH range of 4.5 to 6.0. It was found that precipitation quickly followed the addition of methenamine to solutions of the sulfonamide. Since methenamine is hydrolyzed to formaldehyde in acidic solution, the aldehyde was tested in the same manner. Formaldehyde precipitated sulfamethizole from solution.

Table I summarizes an experiment involving the addition of formaldehyde, methenamine, and mandelic acid to saturated aqueous solutions of sulfamethizole at pH 5.0 and pH 6.0. The buffer systems employed were 0.1 *M* acetate (pH 5.0) and 0.1 *M* phosphate (pH 6.0). A saturated solution of the sulfonamide was prepared in each buffer at room temperature and the actual concentrations of sulfamethizole were determined by assay (2). [Using a Coleman Junior spectrophotometer and selected round cells as described previously (4), the optical density value corresponding to 1 mcg. of sulfamethizole per milliliter was 0.098.] Ten-milliliter aliquots of each sulfonamide solution were treated with stoichiometric quantities of mandelic acid and methenamine and with 6 molar equivalents of formaldehyde. Each mixture was filtered at the end of 1 hour and the filtrates were analyzed for sulfamethizole. At both pH levels, sulfamethizole was precipitated quantitatively by formaldehyde in 1 hour. Methenamine caused the precipitation of 22–33% of the sulfamethizole in 1 hour. In a similar experiment conducted at pH 6.0, methenamine was found to precipitate 65–80% of the sulfamethizole in 20 hours.

Druey (5) and Basu (6) independently discovered that sulfathiazole formed the same insoluble condensation product with either formaldehyde or methenamine. From elemental analyses, both workers suggested possible chemical structures for the product. Later, however, new and polymeric structures were advanced for formosulfathiazole (7, 8). An investigation of the nature of the insoluble sulfamethizole-formaldehyde product is in progress at this laboratory. Another area of interest is the determination of the site of formation of the condensation product (kidney, ureter, bladder).

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Table I.—Precipitation of Sulfamethizole with Formaldehyde and Methenamine

Sulfamethizole, mg./10 ml.	pH	Mandelic Acid, mg.	Methenamine, mg.	Formaldehyde, mg.	Sulfonamide Precipitated, %
6.08	5.0	3.41	...	...	0
6.08	5.0	...	3.14	...	27
6.08	5.0	...	...	4.08	100
6.08	5.0	3.41	3.14	...	25
16.32	6.0	9.17	...	...	0
16.32	6.0	...	8.44	...	22
16.32	6.0	...	...	11.0	99
16.32	6.0	9.17	8.44	...	33

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The Editor Comments

FOR A SOPHISTICATED PUBLIC

The pharmaceutical industry can be rightly proud of the extensive steps it takes in testing new drugs to assess their degree of safety or lack of toxicity. While much of this information or testing has been required under the new drug requirements of the federal law, nevertheless, many firms have generally exceeded the minimum testing required in order to provide as much assurance as possible that a particular drug is adequately safe for use in the treatment of a particular illness.

Unfortunately, however, on the basis of the above experimentation many pharmaceutical firms will refer to their drugs as "*safe*" in promotion campaigns to physicians, pharmacists, and even to the public. In our opinion, this is most unfortunate and ill-advised, since no drug is completely "safe" and without some possible risk associated with its use or misuse.

Other industries, such as the automobile manufacturers and the electrical appliance producers, have long ago recognized that under certain conditions their products might result in injury to the public using them. This realistic attitude has been reflected in their advertising which rarely, if ever, refers to the safety or complete lack of hazard in using their products.

Why is it, then, that the pharmaceutical industry feels that its products must be held out as being completely safe before those products will gain acceptance and use by the health professions or the public? It appears to us that the pharmaceutical industry might well consider directing more of its advertising and promotion resources toward education of the public to the fact that any drug—like everything else we use in everyday life—of necessity has a degree of fundamental hazard connected with it. The sooner that this basic fact is brought home to all those using drugs, the sooner we will have a sophisticated public utilizing the benefits of drugs with a proper realization of the cautions which must be exercised and the potential dangers which cannot be completely divorced from any drug therapy.

Edward G. Feldmann

JOURNAL OF **Pharmaceutical Sciences**

February 1963 volume 52, number 2

Review Article

Plastics in Pharmaceutical Practice and Related Fields. Part II

By JOHN AUTIAN

CONTENTS

[Contents for Part I of this review appeared in the January issue; THIS JOURNAL, 52, 1(1963).]

Leaching

A CONSTITUENT which migrates from a plastic material into a drug system or into a biological system may, for our purposes, be headed under the term *leaching*. In a résumé of this subject, it might be convenient to consider plastic devices or items in two categories. The first of these are plastic materials which can be shaped or composed into a device without the addition of other ingredients. These may be referred to as *pure* plastics, keeping in mind that pure will mean a material composed of only the polymer or copolymer. The second class or category can be called *compounded plastics*, and these materials will be composed

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of the polymer or copolymer with the addition of other ingredients.

Pure Plastics.—The pure plastics should present few problems to the pharmaceutical or medical practitioners in regard to leaching. That there never may be problems, however, is too much to expect even from the so-called pure plastics. The reason will become obvious when it is mentioned that often ingredients are added in very small quantities to stabilize or impart a specific property to the plastic. Since these ingredients are in very small quantities, they often are not revealed as being in the plastic. Other ingredients, such as mold release agents, or even contaminants, can become part of the plastic during the history of manufacture of the particular item. For example, it has been found that when used plastics are reground in a worn-out machine, trace metal contaminants can appear in the final plastic item. On occasions, there may be the possibility of using several pure plastic materials from different sources. Usually, these plastics are formed in pellet or powder form for convenience of use to the manufacturer of the plastic item. One supplier may add a stabilizer to his pellets or powder, which is not added to the material from the second supplier. Other situations could be revealed which would strongly suggest that the pure plastic is not in reality as pure as is believed. These trace amounts of other ingredients could cause some problems to either a drug system, to blood, or to a biological system. In the past few years reports, unfortunately not well documented, showed certain unusual results when plastic Petri dishes were employed in a laboratory (79). A change to another plastic dish, from another company, produced results which were more in keeping with the behavior of the organism.

Untoward problems may arise with the pure plastics when colorants are added to the formula. Even though these colorants might be in relatively small concentrations, the particular dye might migrate into a solution and cause a toxic effect. An example of this was seen by Autian and Brewer (80) in the development of new plastic-hubbed hypodermic needles. They noted that certain of the formulations would release a constituent to saline solution which, when injected into the mice, would cause death. Further investigation of this problem revealed that certain of the colorants could cause the toxic response.

Compounded Plastics.—As has been indicated in the first portion of this review, certain advantages may be gained by the addition of other ingredients to a polymer material. Perhaps the most important property to be gained is flexibility which, of course, would seem to be requisite for tubings which are to be used either to collect body fluids or to transfer drug solutions, blood, and other liquids into the body. The plastic which has found the most use as tubing material is polyvinyl chloride. A properly formulated polyvinyl chloride contains a plasticizer, stabilizing agent, antioxidant, and perhaps other ingredients such as a colorant. Often the final material is composed of 40 to 60% of the other ingredients, primarily of the plasticizer. The presently used polyvinyl chloride tubings for various purposes in medical practice may represent a great many formulas, without the actual formulation being revealed. This, of course, is regrettable since information of this type would prove exceedingly helpful to those who are interested in evaluating the merits of a particular polyvinyl chloride device.

One wonders how much thought was given to the use of compounded plastic materials for those devices which are to have contact with various drug systems or body tissues. The very prospect of migration of one or more ingredients from the material to a solution or tissue would seem to preclude the use of these materials until satisfactory tests are first performed. It is to the credit of several responsible firms that proper evaluations were first conducted prior to the introduction of these items to medical practice. One must, however, always be alert to the possibility of untoward reactions or problems when compounded plastics are employed.

Autian and Kapadia (81) noted that various polyvinyl chloride tubings used for medical purposes would release a constituent to several solvent systems employed in parenteral preparations. A revealing study was made by Thomas and Lagrange (82) on the leaching characteristics of several plastic materials. They exposed these materials to sodium chloride injection and to dextrose injection for a period of time and found that the polyvinyl chloride released a significant quantity of one or more ingredients to the solution. On further analysis, they found that a plasticizer and a stabilizer were the leached constituents. Hemolytic studies revealed that an organo-tin compound, used as the stabilizer, caused the reaction while the plasticizer showed no ill effect on the cells. The question of toxicity due to leaching from plastics was brought to the attention of Meyler, Willebrands, and Durrer (83), who observed

an unusual reaction during a heart perfusion study. The event was noticed directly after plastic tubings were substituted for the conventional glass. In this particular instance, the plastic tubing was a brand of polyvinyl chloride which released a constituent to a perfusion liquid, which in turn caused a cardiotoxic response to an animal heart. Further study of the problem led to the conclusion that the plasticizer was not the culprit, but rather a stabilizer used in the formulation of the plastic tubing.

A recent study in the author's own laboratory should be of interest to the reader for it does demonstrate that even several of the present tubings might cause a toxic effect, if not properly used. In this particular study, a number of unit packaged tubings (most were of the vinyl type) used for various purposes in hospitals were obtained. Many of these tubings were labeled as sterile, pyrogen free, and several indicated that they had been animal tested. Samples of these tubings were implanted into the muscles of rabbits, as suggested by Brewer and Bryant (84), and after from 3 to 7 days they were sacrificed and the area of the implants morphologically examined for any tissue response. Several of these tubings revealed a striking effect upon the tissue which could not be easily overlooked. Gas chromatographic techniques indicated that a number of components would be released to a solvent system composed of alcohol-water systems. Up to this writing, the actual component causing the toxic effect has not been found, but it appears that it may be due to one of the stabilizers. There appears to be little published information on intravenous, subcutaneous, and intramuscular toxicity of the various plasticizers, stabilizers, and antioxidants currently being used in the formulation of polyvinyl chloride tubings. It should be mentioned, however, that most of these ingredients have passed the requirements under the new food additive amendment and are considered nontoxic compounds. But, one should keep in mind that these tests include oral feeding tests and not tissue study tests. This last statement should perhaps be taken with some seriousness since it should dispel the idea that "FDA Approved," at the present time, means that the particular plastic device has been approved by the FDA. Rather, it should be noted that the ingredients used in the device have been approved for a food packaging application, not for a medical application.

From what has been said on leaching, it will be obvious that the solvent can have an appreciable influence on the migration of an ingredient from the plastic to the liquid phase. Further complications can develop when one or more solutes are present in the solvent. One example may illustrate this point. A polyvinyl chloride sheet was exposed to various concentrations of benzalkonium chloride (85). The results indicated that the cationic agent was sorbed by the sheet, but during the sorption process an ingredient or ingredients was being released to the solution. Some thought should also be given to the pH of the solution since experimental evidence has demonstrated that the rate of release might be somewhat dependent upon the hydrogen ion concentration of the surrounding liquid.

Sorption

Introduction.—When a solution is in contact with a solid phase, both the solvent and solute molecules will be striking the solid surface. If the conditions are optimum, both types of molecules may be adsorbed to the surface of the solid. Normally with "nonreactive" materials the quantity of molecules, either solvent or solute, will be negligible and for all practical purposes it may be assumed that no adsorption has taken place. On the other hand, if the chemical structures of the components making up the solid phase are of such a nature that they can electrically attract a molecule in the solution with comparative ease, a situation may exist (depending upon the total surface area of the solid phase exposed to the solution) where a significant quantity of the solute might be removed from the solution. With plastic materials there is usually not only surface attachment, but actual penetration of the solute molecules which in turn come into contact with particular attraction sites in the polymer. In fact, it may be assumed that the greatest extent of uptake of the solute from a solution would be a consequence of the solute penetrating and diffusing into the plastic. This of course, without describing any mechanism of interaction, is exactly what occurs with most dyes in both the natural and synthetic yarns. Drug uptake or binding to a particular plastic can be considered as following one of the many postulated mechanisms for dye-yarn interaction. In general, the interaction is a physicochemical phenomenon, directly related to the chemical structure of the drug and the particular physical and chemical properties of the plastic. A number of other factors will materially affect the uptake and it is well to mention the more important ones:

concentration of solution, particular solvent system, other agents in the solution, pH of the solution, temperature, time of contact, quantity of plastic exposed to solution, purity of plastic, and possible changes in the plastic material after exposure to solution.

To prevent confusion, the term *sorption* will be used in this section to indicate that both adsorption and absorption are taking place, even though one may predominate. The terms *uptake* and *binding* will be used with the understanding that both terms may refer to either equilibrium or nonequilibrium conditions.

It has already been demonstrated that plastic materials are composed of crystalline regions, dispersed throughout amorphous regions. Under the discussion on permeation of gases, the point was clearly emphasized that the penetrant molecule probably could not travel through the crystalline region, but must find passageway in the amorphous zone. Solute molecules in a solution differ not from the picture given for the gas molecules and it may be assumed, except in unusual instances, that these molecules (solute) can travel or diffuse only through the amorphous zone where they become affixed to a particular site in the polymer.

One general method of studying solute-plastic interactions is by the measurement of the quantity of solute taken up at equilibrium by a plastic material at a constant temperature and pressure. The resultant data may then be fitted to one of two basic expressions. The first of these is the empirical relationship of Freundlich (86), which may for this purpose be written as

$$\log q = \log k + 1/n \log C \qquad \text{(Eq. 13)}$$

where q is the quantity of drug or other chemical agents sorbed by a specified quantity of plastic, k and n are constants, and C the equilibrium concentration of the agent in the solution. The second, based upon kinetic considerations by Langmuir (87), may be depicted as shown below

$$\frac{1}{q} = \frac{1}{kSC} + \frac{1}{S} \qquad \text{(Eq. 14)}$$

where k is constant and S may be considered as the theoretical saturation value of solute which will be sorbed by the plastic. A plot of $1/q$ *vs.* $1/C$ will produce a straight line from which the intercept, $1/S$, may be found (see Fig. 9).

Often it is highly desirable to express in a quantitative manner the affinity of a particular drug or agent for a plastic material. In this

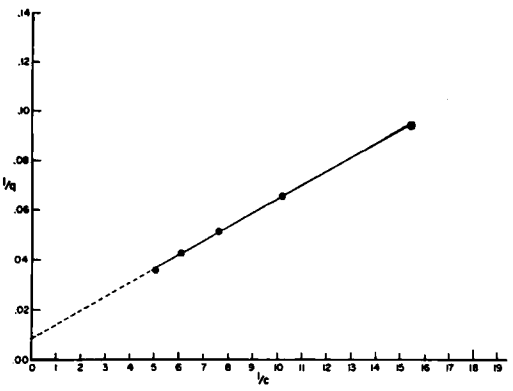

Fig. 9.—A Langmuir plot of sorption data of salicylic acid by nylon at 41.5° C. [From: Kapadia, A. J., Guess, W. L., and Autian, J., "Sorption and Diffusion Studies of Salicylic Acid in a Nylon Sheet," to be published].

instance recourse has been made to the partial molar-free energy or chemical potential (μ). For example, it should be clear that if the chemical potential of a solute in one phase is greater than the potential in a second phase, there will be a driving force moving the solute molecules from the higher to the lower phase. At equilibrium, both chemical potentials would be equal and the driving force would be reduced to zero. It is possible to express the chemical potential of solute as (88)

$$\mu = A + RT \ln a \qquad \text{(Eq. 15)}$$

where A is a constant, dependent upon the temperature and pressure but independent of composition, R and T the usual symbols for the gas constant and absolute temperature, while a is the activity of the solute. By comparing the chemical potential in any given state to that in a standard state, represented as follows

$$\mu^o = A + RT \ln a^o \qquad \text{(Eq. 16)}$$

and subtracting this last equation from the previous one will give

$$\mu - \mu^o = RT \ln a/a^o \qquad \text{(Eq. 17)}$$

Now if in the standard state a^0 is given the value of unity, Eq. 17 will reduce to

$$\mu = \mu^o + RT \ln a$$

If we now consider a drug or solute distributed between a liquid phase (in solution) and a solid phase (plastic), we can write the expression depicting the conditions in both phases as

$$\mu_l = \mu_l^o + RT \ln a_l \quad \text{(for liquid phase)} \qquad \text{(Eq. 18)}$$
$$\mu_s = \mu_s^o + RT \ln a_s \quad \text{(solid phase)}$$

but at equilibrium

$$\mu_1 = \mu_s$$

or

$$-\Delta\mu^o = RT \ln a_s/a_1 \qquad \text{(Eq. 19)}$$

If it is assumed that the solute forms an ideal solution in both phases, activities may be replaced by concentration or

$$-\Delta\mu^o = RT \ln C_s/C_1 \qquad \text{(Eq. 20)}$$

The difference in standard chemical potential ($\Delta\mu^o$) may now be considered as the quantitative measure of the tendency of a drug or solute to move from its standard state in a liquid to its standard state in the plastic. Furthermore, this standard chemical potential may be taken as the affinity of the drug or other agent toward a particular plastic.

Equation 20 is based upon ideal conditions, a state which is often difficult to attain in practice and for this reason other more complex expressions have been developed for the evaluation of the affinity. It also appears that each particular plastic material might require a separate equation. Gilbert and Rideal (89) were one of the first to formulate a mathematical expression which could be applied to a practical situation; in this case the interaction of mineral acids with insoluble fibrous materials. Others have since used the Gilbert and Rideal equation directly, or have modified it, to fit their particular needs. Vickerstaff reviews a number of these expressions, based upon the postulated mechanism of interaction, and the reader should refer to his text for further information (90).

Little, if any, data have been published on the affinities of various agents to plastic materials, but considerable data exist for various dyes and textile material (90). Unfortunately, some of these data are in error due to the collection of results at conditions other than at equilibrium. It would seem, however, that the standard chemical potential could be used to indicate the affinity of a drug to a specific plastic material if and when such information becomes of sufficient interest to those in the pharmaceutical or research field.

Negligible uptake or sorption will be noted, at least by conventional analytical means, for a number of drugs and plastic materials. For example, with our present information it can be assumed that little sorption will occur with those plastic materials which do not have polar sites, the possibility of high uptake or sorption are minimal and often not easily discerned unless very large surface areas are exposed to the solution. Significant sorption will, however, be seen for those hydrophilic plastic materials (nylon, cellulose, acetates, etc.) which will permit the diffusion of the solute. As might be anticipated, the physical and chemical properties of the solute will also be a determinant for sorption for it appears likely that the interaction between a solute molecule and the polymer is one of an electrical nature, whether it be one of ion-ion type, dipole-dipole, ion-dipole or through secondary valence forces, such as Van der Waals' forces, or perhaps a combination of these.

In order to represent the sorption process in a simple manner, Fig. 10 is presented. Here it may be seen that a drug molecule, D, is in solution in contact with a polar plastic material. The parallel lines in the plastic material represent the crystalline zone or region, while the lower lines attempt to portray an amorphous region in the same plastic. Sites for possible interaction in the polymers are shown by the dots. When the solute molecules are placed into the solvent, one or more will diffuse toward the surface of the plastic and become adsorbed. When most of these surface sites are filled, there will be sufficient energy to permit these solute molecules to penetrate the surface and to travel or diffuse into the amorphous zone of the plastic where new binding sites become available. When all available sites are filled, equilibrium has been reached and sorption attained at one specific temperature and pressure. In most instances it will be correct to assume that the rate determining step of the sorption process is the diffusion of the drug in the matrix of the plastic and if this is the case, then special emphasis must be placed upon the diffusion constant or coefficient.

Diffusion.—In reviewing, even briefly, aspects of diffusion in insoluble materials such as plastics, it will be necessary once again to allude to some of the work of dyers and colorants in the textile industry, since this group by far has been concerned to a greater degree and depth in this facet of research than perhaps any other single group.

The usual experiments which have been utilized for the study of diffusion of solute molecules in insoluble polymeric materials may be divided into two general types: studies under a steady-state condition, and studies under nonsteady-state conditions.

Perhaps the simplest method of evaluating the diffusion coefficient of a solute in a plastic is to set up an experiment under the steady-state condition. In one compartment a known

solution of the particular solute is placed, while in the other an equal quantity of water. At repeated intervals, the water compartment is measured for solute which has permeated through the film. The data are then plotted as concentration of drug or agent appearing in the water compartment against time. The curve will initially curve, but after a steady-state condition is reached, a linear relationship will be observed from which the diffusion coefficient may be calculated using the familiar time-lag equation already given under permeation

$$D = \frac{l^2}{6\tau} \qquad \text{(Eq. 21)}$$

where l is the thickness of the film and τ the time lag or the point where the straight portion of the curve crosses the time axis (see Fig. 4).

Even though the steady-state condition has much to be desired in evaluating diffusion coefficients, often it becomes impossible to use this method and one must therefore turn his attention to experiments where nonsteady-state conditions exist. Under the nonsteady-state conditions, where the concentration of the solute in the plastic is continually changing, Fick's second law is applicable or

$$\frac{dC}{dt} = D\frac{d^2C}{dx^2} \qquad \text{(Eq. 22)}$$

where C is the concentration, D, t, and x are terms already defined. It is not, however, possible to solve for D in the above equation directly and hence recourse must be made to other mathematical expressions which are in themselves special cases dependent upon the experimental design. These special cases, for convenience, may be divided into two broad categories: for "finite solutions" (or where the concentration of the drug in the solution does not appreciably decrease over the time period of the experiment), and for "infinite solutions" (or where the concentration of the drug in the solution does decrease over the time period of the experiment). Each of these categories may be further divided into the particular geometry of the plastic (*i.e.*, plane sheet, cylinder, or sphere) and whether the diffusion coefficient is constant or varied over various concentration ranges of the solute in the plastic. Representa-

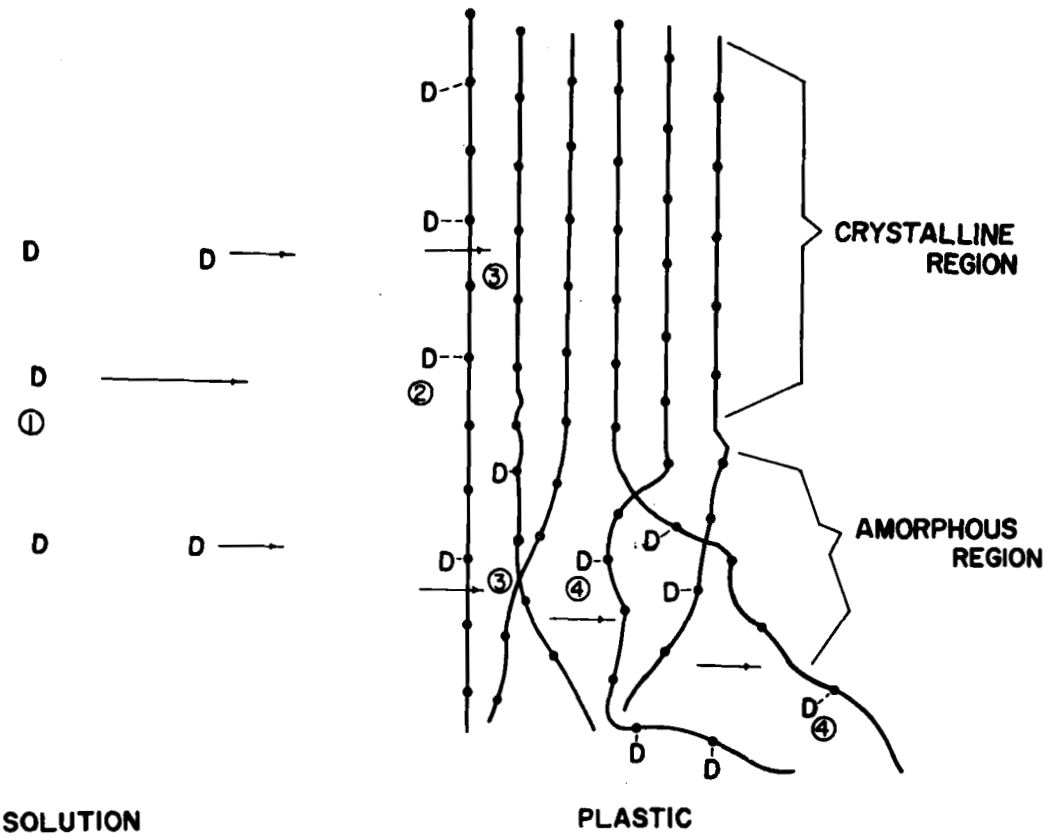

Fig. 10.—A schematic model showing sorption of a drug by nylon. D = drug molecule; -- = polymer chain; and • = binding sites in polymer.

tive mathematical expressions will be given for several of these special cases in the section to follow, but for more details the reader should become familiar with several texts on the subject of diffusion, in particular the books of Barrer (91), Jost (92), and Crank (93).

Perhaps the most work in the past for the calculation of diffusion coefficients has been based upon the "infinite solution" and a diffusion coefficient which may be considered as constant, an assumption which may not be correct, as we will learn later. For simplicity also, the plane sheet has most often been employed, even though the cylinder and sphere have received attention. For the most part, the mathematical expressions or methods have been derived for use with data collected from sorption studies.

McBain (94), as early as 1909, used an expression which apparently was borrowed from previous investigators on heat transfer problems to evaluate D from adsorption data. The equation may be stated as

$$\frac{M_t}{M_\infty} = 1 - \frac{8}{\pi^2}\left(e^{-\pi^2 Dt/l^2} + \frac{1}{9}e^{-9\pi^2 Dt/l^2} + \frac{1}{25}e^{-25\pi^2 Dt/l^2} + \ldots\right) \quad \text{(Eq. 23)}$$

where M_t is the concentration of drug or agent taken up by a sheet in time t, M_∞ is amount of the drug or agent taken up at equilibrium, and l is the thickness of the sheet. Hill in 1928 (95) used a nearly similar equation to calculate the diffusion of oxygen into muscle tissue and this particular equation has since been suggested for calculation of diffusion into cylinders.

Neal and Stringfellow (96) used Eq. 23 for the first time to calculate the diffusion coefficient of a dye in cellulose from absorption-time data. Crank and Henry (97) presented an expression following an infinite series as shown above for calculating D. This expression follows

$$\frac{M_t}{M_\infty} = 1 - \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2\pi^2} \times e^{-D_o\pi^2(n+1/2)^2 t/l^2}, \ldots \quad \text{(Eq. 24)}$$

For simplicity it is best to find a suitable value for t/l^2 for which $M_t/M_\infty = 1/2$ or

$$\left(\frac{t}{l^2}\right)_{1/2} = -\frac{1}{\pi^2 D_o} \ln\left\{\frac{\pi^2}{16} - \frac{1}{9}\left(\frac{\pi^2}{16}\right)^9\right\}, \ldots \quad \text{(Eq. 25)}$$

which may be reduced to

$$D_o = 0.04939/(t/l^2)_{1/2} \ldots \quad \text{(Eq. 26)}$$

Equation 26 can be used rather conveniently to find D. A sorption-time experiment is run from which t at 50% sorption is found. Since t and l (thickness) are known, D may be calculated. Up to this point it has been assumed that the diffusion coefficient was a constant and not dependent upon concentration in the plastic. Recently this has been found not to be the case for many systems and it has been surmised that (97, 98) values for D using the above equations are actually average values and should be indicated as such or designated as apparent diffusion coefficients. Crank and Park (99), using Eq. 26 as a starting point, developed a method of evaluating D as a function of concentration. These same authors have also found that desorption studies may also be used for the calculation of D and that if both are considered, a better approximation for D (than by either sorption or desorption) is possible.

In the past also, it has usually been necessary to run sorption experiments in such a manner that the concentration of the drug or agent in the solution would remain nearly constant. This problem has now been overcome by the development of newer mathematical expressions for "finite solutions" or for those cases where there is a decrease in the concentration of solution over the time period for equilibrium to be reached. Crank (93) gives full treatment to these methods in his excellent text on the mathematics of diffusion.

Since McBain's equation, a great many other equations of the same general type have evolved for both "infinite" and "finite" solutions. These usually have followed an infinite series which might be represented by the general expression (100)

$$\frac{M_t}{M_\infty} = 1 - A.e^{-BK} - C.e^{-FK} - G.e^{-HK} \ldots \quad \text{(Eq. 27)}$$

where A, B, C, etc., are numerical constants of known values and K is equal to Dt/l^2 for a plane sheet (or if for a cylinder l can be considered as the radius). The task of solving equations of this type are rather formidable and quite time consuming. Fortunately, the task has been simplified by preparing theoretical values of M_t/M_∞ for various values of K. From these tables it becomes an easy task to evaluate the apparent diffusion coefficients, as is now described. First, a sorption experiment is run at a specified temperature and the data plotted as M_t/M_∞ *vs.* the square root of time. If the diffusion is of a Fickian nature, a straight line relation will result.

Now a theoretical plot is made of M_t/M_∞ *vs.* the square root of K from data taken from

the appropriate table. This relationship will also result in a straight line. From the experimental curve at a definite M_t/M_∞ value, the appropriate t is found. In an identical manner and at the same M_t/M_∞ value, K is deduced. Since $K = Dt/l^2$ and since the values for K, t, and l are now known, it is a simple calculation to find the diffusion coefficient.

This graphical method using appropriate tables has had great use, particularly in evaluating the diffusion of dyes in textiles. Since very little published work is available for the diffusion of drugs in plastics, only one example will be cited to demonstrate the ease with which these tables can be used. The particular case will be the work of Kapadia, *et al.* (101), who studied the diffusion of salicylic acid in nylon. Their particular experiment was of the "finite solution" type, using a plane sheet.

Sorption studies were conducted at various temperatures and a plot was made of M_t/M_∞ *vs.* $\sqrt{t}$ as shown in Fig. 11. For each temperature the total amount of drug removed from the solution at equilibrium was found and equated to a per cent value. From Bertheir's table (see Fig. 12) and at the appropriate total per cent uptake

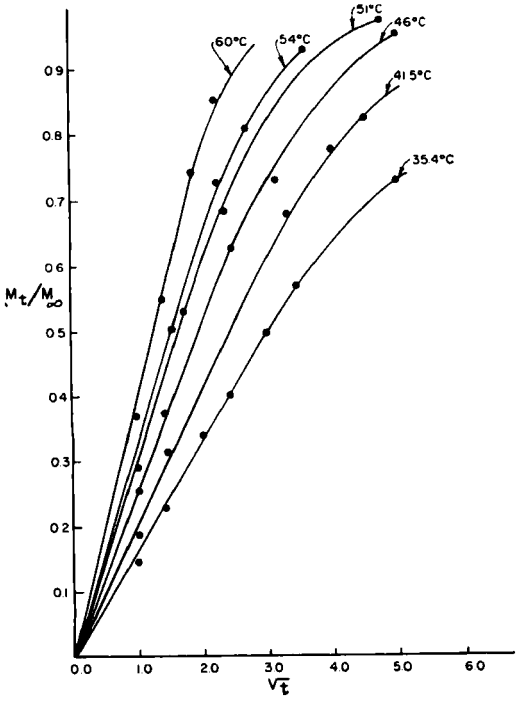

Fig. 11.—Fractional uptake of salicylic acid *vs.* square root of time at various temperatures. [From Kapadia, A. J., Guess, W. L., and Autian, J., "Sorption and Diffusion Studies of Salicylic Acid in a Nylon Sheet," to be published.]

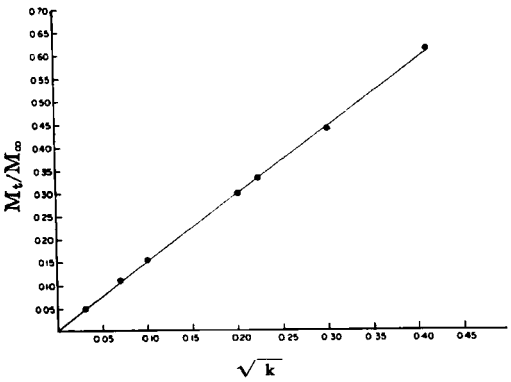

Fig. 12.—Theoretical plot of fractional uptake *vs.* the square root of K at a total per cent uptake of 45.8. K is taken from Berthier's table for a plane sheet. [Berthier, G., *J. Chim. Phys.*, **49**, 527(1952).]

TABLE VIII.—APPARENT DIFFUSION COEFFICIENT OF SALICYLIC ACID IN NYLON AT VARIOUS TEMPERATURES[a]

Temperature, °C.	D, cm.²/sec.
35.4	8.97×10^{-10}
41.5	1.26×10^{-9}
46.0	2.08×10^{-9}
51.0	2.73×10^{-9}
54.0	3.86×10^{-9}
60.0	5.91×10^{-9}

[a] Kapadia, A. J., Guess, W. L., and Autian, J., to be published.

(at equilibrium), corresponding K values for each M_t/M_∞ were used to draw a theoretical curve as shown in Fig. 12. In the manner already described, or by the method of slopes, the apparent diffusion for each temperature was calculated. These values are included in Table VIII.

Some remarks should be made in regards to the variable diffusion coefficients. Even though expressions have been developed for calculating these values as a function of concentration in the plastic, it becomes a bit more complex to arrange the experiment where accurate values of the concentration in a particular point of the plastic can be measured. McGregor, Peters, and Petropoulos in recent publications point out some of the fallacies inherent with previously used methods for studying the variable diffusion coefficients and recommend a microdensitometric technique for more accurate values of these constants (102–104). For extremely accurate work and where fundamental knowledge may be needed in regards to the diffusion of a drug into a solid material, the method of McGregor, *et al.* (102), may hold great promise. The exact technique, however,

will undoubtedly pose some problems which may be difficult to overcome in certain laboratories.

Mention might also be made of the advantage of using radiotracer techniques in diffusion studies. For example, Hayashi (105) has found this method as a very fast method for determining the diffusion of surface-active agents in nylon. Briner, *et al.* (106), have employed a liquid scintillation method for measuring the diffusion of certain agents in plastics. The radiotracer method makes possible the evaluation of diffusion in a substrate, which might not be feasible by the use of conventional analytical methods and it undoubtedly is a method which will receive greater attention in the very near future.

Mechanism for Sorption and Factors Influencing the Process.—*General.*—It has been pointed out in this review, and demonstrated experimentally in many instances, that diffusion of the solute in the matrix of the plastic is the rate determining step in the overall process of sorption. Here it should not be implied that little importance is attached to those instances when only surface adsorption occurs, but rather to indicate that when both adsorption (at surface) and absorption are taking place, most likely the quantity of solute adsorbed is insignificant to the amount absorbed. That serious consequences might arise when a very potent drug or an extremely active biological agent is adsorbed on the surface of a plastic, even in microquantities, is certainly not to be minimized and good pharmaceutical practice demands an appreciation of this fact.

The question now arises as to how best depict a mechanism for sorption of an agent by a plastic material. This task has presented a formidable obstacle to numerous conscientious investigators and it appears now that no one mechanism can describe all of the sorption process for all the varieties of plastic materials. In fact, often a number of theories can be used to describe the same interaction between a solute and a plastic. With our present knowledge, one must be content to accept certain, rather broad, generalities on the mechanism of sorption and then to turn his attention to a number of factors which may influence the sorption process.

Even though the uptake of a solute from a solution onto and into a plastic material is a sorption phenomenon, it should be clear that the chemical structure of both the solute and the polymer (making up the plastic) are the determining factors for sorption to occur. A second consideration, which can play a rather large role in the sorption process, is the physical condition of the particular plastic material. Here reference is made to the available regions in the plastic which will permit entrance and travel to the solute molecules, for without these passageways the solute will react only at the surface of the material. All other factors, such as pH, solvent systems, concentration of solute, presence of other ingredients in both the solution and plastic, and temperature only diminish or accent the magnitude of sorption.

Effect of Structure.—In considering structural relationship and uptake of drugs by plastic, one soon recognizes that little available literature has been compiled on this subject by the pharmaceutical and medical professions, the obvious reason being that sorption of a drug is highly undesired, and all efforts will be expended to prevent such an incident from occurring. One must, therefore, dip into the literature of other disciplines, in particular the textile group, to learn of the influence of the structure on uptake of chemical agents, primarily those which are used for dying of yarns (90). Other related data may be sifted from extensive work in the biological field on the binding of various agents to proteins (107–110) and protein-like compounds and recent contributions from the physical pharmacists on binding of drugs with macromolecules used in pharmaceutical systems (111–114). Data from the textile industry seem to be most closely related to our particular interest because textiles both chemically and physically resemble the plastic materials which are used in pharmacy and medicine and because textiles are solid materials, whereas the polymeric materials of the biochemists and the physical pharmacist have been primarily of the water-soluble type.

The greatest bulk of substrates investigated by the textile group has been those yarns which can be either of the cellulosic type (cotton, cellophane, cellulose acetate) or the polyamide type (silk, wool, and nylon). From these two types of materials and with the addition of other information, an attempt will be made to view certain structure features of the solute molecules which give rise to the interaction (and of sorption) between an agent and a polymeric material.

One important feature of a dye molecule is its ionic charge which can arise from such functional groups as the sulfo and carboxyl radicals for acidic dyes and from cationic quaternary

ammonium groups in basic dyes (115). The presence of this electrical charge, positive or negative, can cause either an interaction to take place or conversely can prevent an interaction. Other forces beside the ionic type for attracting and binding solute molecules to a polymer have also been recognized and it appears that the particular substrate will have a further influence on which force or combination of forces will support the interaction. For example, wool (a polyamide-like material) seems to have the ability of attracting any type of solute molecule, which will ionize in solution, while for substrates such as cellulose acetates or certain of the polyesters (Orlon), little uptake will be noted for those solute molecules which are in ionic form (115).

It seems to be well established that for cellulose fibers, the charge on the solute is of secondary importance and the particular structure and the geometric arrangement in the molecule are the most critical points in the dyeing or sorption process. A number of investigators have indicated that the dye molecule must be linear so that it can lie parallel to the polymer chain and further, that the molecule (dye) should contain polar groups spaced at critical intervals to interact with specific sites in the cellulose polymers (116–118). Vickerstaff points out that the dye-cellulose interaction seems to favor a "lock and key" process (115).

From the great number of studies on the dyeing of cellulose, Vickerstaff has been able to set down a number of requirements which a dye should meet in order to be considered for dyeing of cellulose material. These may be stated as follows (119):

1. The molecule should be capable of assuming a linear configuration.
2. The aromatic nuclei should be capable of assuming a coplanar arrangement.
3. The molecule should contain groups capable of forming hydrogen bonds.
4. The presence of a conjugated system of double bonds, which by resonance promotes coplanarity of the molecule and probably favors hydrogen bond formation by the groups at the ends of the conjugated chain.

Favorable, but not essential, conditions are (again from Vickerstaff).

5. Widely spaced hydrogen bond-forming groups.
6. The presence of the minimum number of solubilizing groups necessary for solubility.
7. The disposition of the solubilizing groups along one side of the dye molecule and of hydrogen bonding groups along the other side.

Until recently, little work of a physical chemical nature was conducted on the uptake of surface-active agents by cellulose materials. White and associates (120–122) made an intensive investigation of the absorption of certain cationic surface-active agents by cellulosic substrates (such as cotton). They theorized that the uptake was due to two processes, namely a cationic exchange between the surface-active agent and the polymer followed by an ion-pair absorption.

The acetylation of the hydroxyl groups in cellulose gives rise to commercially important materials such as cellophane and rayon. Alteration in the structure of the original material (cellulose) by the presence of acetyl groups produces a marked difference in the sorption of various solute materials. For example, most anionic dyes will not produce good coloring of rayon material as they will on cotton (123). Several reasons have been given for the inability of cellulose acetate to behave like cellulose (124). For one reason, the hydrophilic characteristic of cellulose acetate is decreased, which in turn prevents swelling (in water) in comparison to cellulose. This affords less opportunity for certain molecules to find passage through the material. A second reason for less uptake of a solute such as the acidic dyes (or anions) is the high negative surface potential on the cellulose acetate which repels the anionic molecules. Conversely, however, the cationic molecules become easily sorbed. Giles (125) has presented an interesting discussion on effects of structure on the dyeing of cellulose acetate. In his paper, he points out that a review of studies on adsorption (including absorption) gives rise to three general mechanisms of interaction: solid solution, polar forces, and nonpolar forces. Rather than coming to the conclusion that these mechanisms are conflicting, Giles gives recognition that all three types may be true, under certain circumstances. Several other interesting points are revealed in Giles' work. For example, as the number of hydrogen donating groups increase in a particular molecule, a general increase in uptake will be noted. There are instances, however, where this rule is not followed, but these exceptions might be explainable on the internal bonding of these functional groups, preventing the hydrogen from the solute molecule to bind with a negative site in the cellulose acetate. Steric hindrance may also block hydrogen donating groups (in the solute molecule) from interacting with the polymer. Forces of attraction may also be set up between hydrogen molecules of the cellulose acetate and negative sites in the solute molecule, forming the usual

hydrogen bond. Well-documented evidence is at hand to demonstrate the effect which the hydrophobic portion of the solute molecule will have on sorption of solute molecules by cellulose acetate and, in fact, with most types of substrates (125). In general, it will follow that in a related series of solute molecules, uptake of the solute will increase with an increase in an aliphatic chain.

Very little published information, if any, has appeared in which the objective of the investigator was to study sorption of drugs by cellulose acetate. Saski (126), however, has recently undertaken such a study and has demonstrated that sorbic acid will be bound to various degrees to the plastic, both from an aqueous and from a hydroalcoholic solution. It would be reasonable to assume that other similar agents would also be sorbed by the cellulose acetate, and it will be interesting to see how structural changes in the solute molecule will alter the overall uptake.

The protein fibers (silk and wool) and the polyamide fibers and plastics (nylon) present a very old substrate (silk and wool) and a relatively new substrate (nylon). In general, the uptake of certain agents on the above mentioned substrates follow to various degrees the same pathways but since nylon is a synthetic material and since more experimental work has been conducted on this substrate, the remaining portion of the section will be devoted to nylon.

Nylon has three polar sites in the molecule. On one end of the molecule there is a carboxylic acid group while on the other there is an amine group. Dispersed throughout the chain there are a number of amide linkages. These polar sites for the main part determine the binding phenomenon. Acidic dyes (salts of the various acid dyes are actually used) will interact with the terminal amino group in the following manner (127, 128). In a pH above 3.0 the end amino group will become positively charged. This positive charge will then bind the anion of the dye. In the stated pH range (above 3.0) there appears to be little interaction at the amide linkages. The carboxylic group seems to contribute little to binding of these acidic dyes. On the other hand, weak organic acids may be bound to both the terminal amino group and to the amide linkages (in this case by hydrogen bonding). Phenol appears to be bound only through hydrogen bonding to the amide linkages since acetylation of the amino group blocks completely the binding (129). As will be seen shortly, the pH of the solution will have a great effect on the uptake of acidic compounds.

Studies in the author's own laboratory have indicated that a great many weak organic acids will be sorbed by nylon (130–132). Those already studied include phenol, salicylic acid, the parabens, sorbic acid, benzoic acid, and derivatives of benzoic acid. Not all weak organic acids are bound, however, as may be witnessed by the work of Briner, Autian, and Skolaut (133) who found that no significant amounts of acetic acid, glycine, or tyrosine were sorbed by nylon. These results give further support to the already well-emphasized fact that with our present knowledge predictions of binding or uptake can only be considered as a hazardous guess—at least with the polyamides.

Cationic agents have been studied as to uptake by nylon and it appears that these interact at the negative sites in the polymer, but the exact mechanism has not been clearly defined. Guess and group (134) noted the uptake of both dicyclomine hydrochloride and benzalkonium chloride. Others have investigated the uptake of surface-active agents of the cationic type and found these also to be sorbed (105, 135).

Steric effects and internal bond formation may reduce the uptake of a particular solute molecule by nylon (as well as other substrates). For example, it has been found that salicylic acid will be bound to nylon in very large quantities, but that acetylsalicylic acid under identical experimental conditions revealed little or no binding (13).

One cannot ignore the influence of van der Waals' forces on the overall interaction between a solute and a polyamide (125, 129, 136). Many examples may be cited to demonstrate that in those molecules which are bound to various substrates, an increase in the hydrophobic characteristic will usually increase the uptake. Practical and theoretical work must still be pursued in regard to nylon as well as to other plastic materials in regard to the influence of the structure of the solute on uptake, in particular, those molecules which resemble or are considered as drugs.

Effect of pH.—Sorption of a particular solute by a plastic material will not only depend upon the structure of the polymer and the solute, but may also depend upon the hydrogen ion concentration of the solution. This of course is true of those agents which might be classified as weak organic acids or weak organic bases. The polyamides (nylon) are a good substrate to illustrate an example where pH influences the

uptake of weak organic acids. A number of textile investigators have observed that acid dyes will have less tendency to be sorbed by nylon as the pH of the dye bath is increased (127, 128). Opinions have been offered as to the mode of interaction and even though there is some difference in opinion among the investigators, Peters' explanation for a number of dyes seems to be fairly well supported by experimental data.

An analysis of an actual nylon molecule would reveal that there are many more amide groups than either of the terminal end groups (carboxylic or amino). At very high pH values, the uptake of an acid dye is quite low, but as the pH decreases a sharp increase in uptake is noted which quickly reaches a fairly constant value. At a pH around 3.0–2.5, another sudden upsurge is noted which continues with further increase in uptake. This S-type of uptake curve appears to be true of most acidic dyes and weak organic acids. Peters (127) has postulated the following mechanism for the pH effect. At the high pH values the interaction of the dye (as an ion) takes place on terminal amino groups which are positively charged forming a salt-type of binding (ion-ion). This type of combination between a dye and a substrate has already been reported for wool (137). Proof that the amino groups are taking part in the interaction may be demonstrated by acetylating these groups in nylon, whereby the uptake at the high pH is decreased. As the pH is decreased, binding or uptake is increased but does reach a limiting value which is interpreted to mean that all of the amino groups have become saturated with the dye. At the lowest pH range 3.0 and below, the nitrogen of the amide group has taken on a positive charge and attracts the dye (which is still in an ionic form even at low pH values).

Some investigators have indicated that the high rate of uptake at low pH values for the acidic dyes are in reality due to the hydrolysis of the main chain which creates new sites for interaction (138, 139). Others (127, 140) have disputed this thesis in the past but the work of O'Briain and Peters (141) sheds considerable light on the actual mechanism of high uptake at low pH values. These latter workers reveal that the acidic dyes are bound to the amide linkages either as free acids or by adsorption of the hydrogen ions to the amides followed by the dye anion. Simultaneously but much slower binding takes place with new terminal amino groups due to hydrolysis of the polymer.

Kapadia, Guess, and Autian (101) have also noted the S-type of sorption curve for salicylic acid. Figure 13 is taken from their study and illustrates the influence pH has on salicylic acid in nylon. Their postulated mechanism at the lower pH is binding of the acid to the amide linkages by hydrogen bonding since at the low pH values the salicylic acid is completely in the unionized form.

Effect of Solvents.—The solvent system may have an appreciable influence on the uptake of solute molecules. Several reasons have been stated for the effect of the solvent on sorption. Perhaps the most important influence the solvent can have is to swell and plasticize the plastic

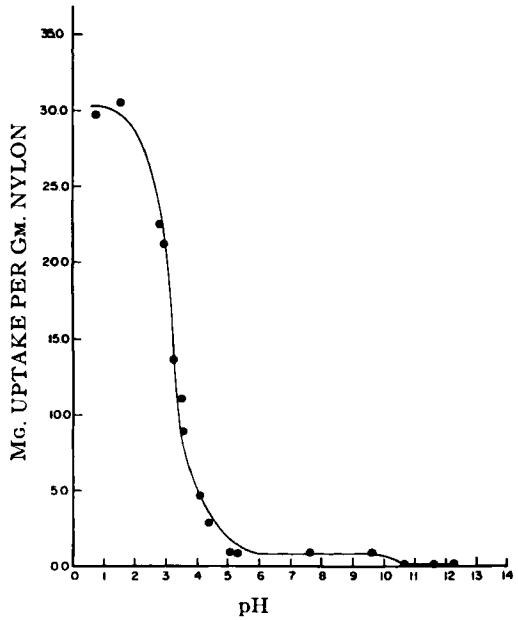

Fig. 13.—Uptake of salicylic acid by nylon at different pH values. [From Kapadia, A. J., Guess, W. L., and Autian, J., "Sorption and Diffusion Studies of Salicylic Acid in a Nylon Sheet," to be published.]

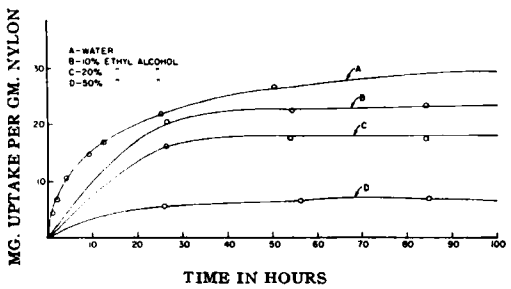

Fig. 14.—Effect of ethyl alcohol on the sorption of salicylic acid (0.15%) by nylon (41.5°C). [From Kapadia, A. J., Guess, W. L., and Autian, J., "Sorption and Diffusion Studies of Salicylic Acid in a Nylon Sheet," to be published.]

permitting more passageways to open up for the solute molecules (142). A less polar solvent system will have less tendency to swell a hydrophilic plastic material, which in turn should decrease the uptake of a solute. One example of this may be seen in Fig. 14 where various concentrations of water-alcohol systems have been used as a solvent for salicylic acid. As the concentration of alcohol increases, there is a general drop in the quantity of drug sorbed at equilibrium by the nylon. This trend may not always be followed. For example, Teulings and White (143) noted that certain dyes (dispersed dyes) would show a decrease in uptake at equilibrium in cellulose acetate up to a specific concentration and then an increase in uptake with an increase in butanol concentration. One should of course keep in mind that certain organic solvent systems may alter the structure of the particular plastic material in a manner which increases the amorphous content of the plastic. This structural change would then make available new sites for the binding of solute molecules.

Effect of Concentration.—Certain unusual features may be met with in regard to the concentration of a particular solute in a solution. As an illustration of this point reference is made to Figs. 15 and 16. In this particular study, the investigators were observing the uptake of several acidic drugs by nylon syringes. Figure 15 demonstrates the usual uptake curve at various concentrations when the data are plotted as bound *vs.* unbound drug (in this case sorbic acid). In like manner, the data for phenol are plotted as appears in Fig. 16. It is evident that phenol, up to a certain concentration, deviates markedly from sorbic acid. In the case of phenol, the uptake increases in a linear fashion but at a specific concentration there is a sudden increase in the uptake. This result may be attributed to the swelling and plasticizing effect which phenol will have on nylon, but apparently this influence is not felt until a critical concentration is approached (130). Other effects due to concentration, which are not as dramatic as phenol, have been observed in careful experiments of certain acidic drugs in nylon. In one particular study it was noted that the Langmuir relation would hold true for a certain concentration range, but an inflection would be seen if all the points were plotted on the curve (101). Much more information will be needed to interpret these results, but it is important to recognize that concentration effects should not be ignored in practical applications, since even slight effects might cause, on long storage conditions, unexplainable results.

Another group of compounds, which have shown unusual behavior in regard to the influence of concentration, are the surface-active compounds on solid substrates. In general, as the concentration of the solution is increased, adsorption or sorption increases in a regular manner until the critical micelle concentration is reached and a sudden increase in uptake is noted. With further increase in the concentration of the surface-active agent, the uptake

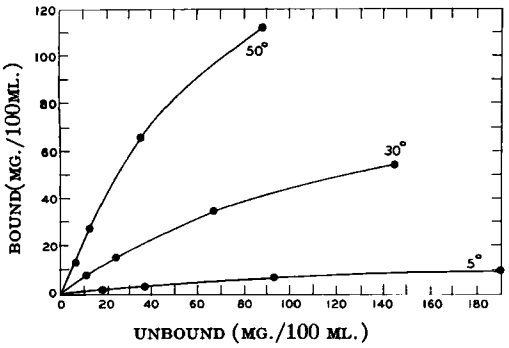

Fig. 15.—Uptake of sorbic acid by nylon syringes. [From Marcus, E., Kim, H. K., and Autian, J., THIS JOURNAL, 48, 457(1959).]

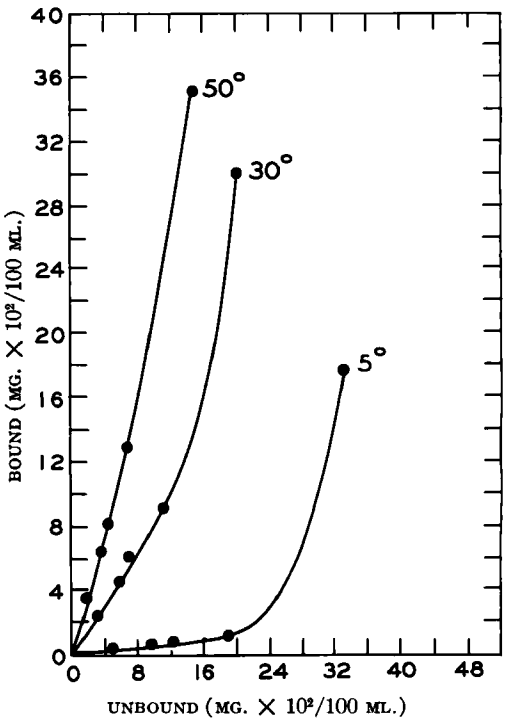

Fig. 16.—Uptake of phenol by nylon syringes. [From Marcus, E., Kim, H. K., and Autian, J., THIS JOURNAL, 48, 457(1959).]

continues until a limiting value is reached. Aickin (144) is believed to have been one of the first to note this type of behavior with surface-active agents, but since then, a great many other investigators have observed the same phenomenon on a number of unrelated substrates. Vold and Sivaramakrishnan (145) explained the sharp increase at the critical micelle concentration of surface-active agents on carbon black as being due to the binding of the surface-active agent as micelles on the substrate, rather than individual molecules. Hayashi (105), in one of his studies on nylon, postulated that below the c.m.c., the uptake was probably due to a chemical adsorption of the surface-active agent on the nylon, but at or above the c.m.c. the agent was adsorbed as micelles.

Effect of Other Ingredients in Solution.—Most practical systems, especially those which might be considered as drug systems, are composed of usually more than one solute and it becomes necessary to establish the influence of these other ingredients on the sorption process. It is known, for example, in the dyeing industry that inclusion of electrolytes will aid in the sorption process (90). Other solute molecules, which may structurally resemble the main ingredient in a solution, are apt to compete for sites in the plastic and in these instances a knowledge of the affinities would be of help in predicting which ingredient would be sorbed to the greatest extent. Possibility of interaction between the same molecules and with other molecules in the solution forming a complex or micelle will undoubtedly have an influence on the uptake, which would be extremely difficult to predict without a thorough study of the problem experimentally.

Effect of Temperature.—Temperature may have two effects on the sorption process. As the temperature is increased, the rate of uptake is increased, but the uptake at equilibrium is decreased. This statement is illustrated in Fig. 17 showing the effect of temperature on the uptake of salicylic acid by nylon. The fast rate of uptake at the higher temperatures is directly related to the increase in the diffusion rate. The lower equilibrium uptake is a consequence of several factors of which the increase in kinetic energy of both the solute molecules and polymer molecules plays the most decisive role. This energy appears to be sufficient to break bond formations between the solute and the polymer which were stable at lower temperatures and also appears to prevent, statistically, adequate solute orientation to the binding sites due to the increase in mobility of both the solute and the polymer. Certain anomalous results have been obtained with cationic agents and nylon. In these cases there was an increase of uptake with an increase of temperature. These values indicate another mechanism for the interaction (134) or a degradation of the polymer.

A great many of the investigated solute-polymer uptake data may be plotted to the Arrhenius equation with considerable success. Since diffusion is considered as the rate determining step, the influence of temperature on diffusion is usually employed as

$$\log D = \log D_o - \Delta E/2.303\ RT$$

where D is the apparent diffusion coefficient

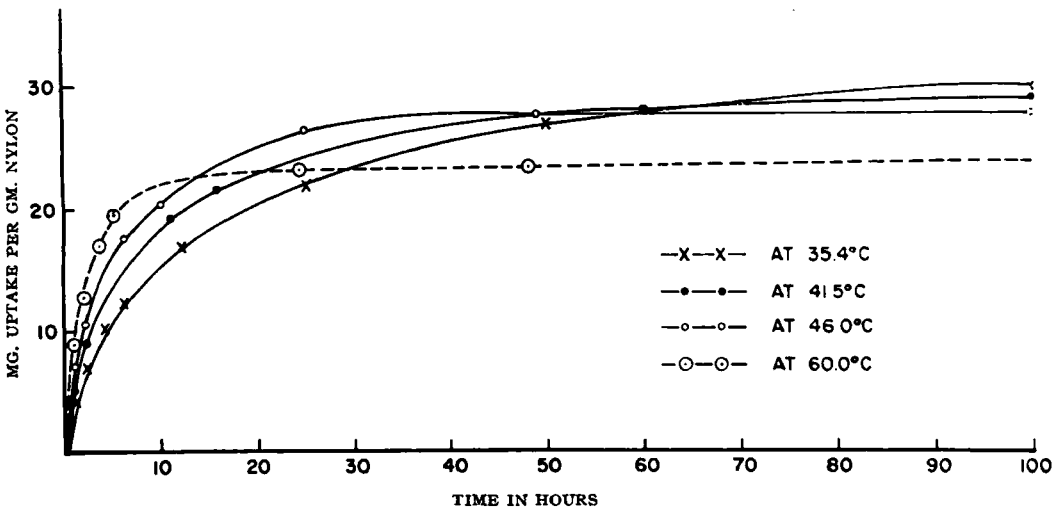

Fig. 17.—Effect of temperature on the sorption of salicylic acid by nylon. [From Kapadia, A. J., Guess, W. L., and Autian, J., "Sorption and Diffusion Studies of Salicylic Acid in a Nylon Sheet," to be published.]

D_o is a pre-exponential factor indicating the value of D at infinite temperature, ΔE the activation energy for diffusion, R and T the appropriate gas constant and absolute temperature, respectively. A plot of log D *vs.* $1/T$ for salicylic acid is shown in Fig. 18 from which the activation energy for diffusion may be calculated and in this case ΔE is 20.5 Kcal./mole.

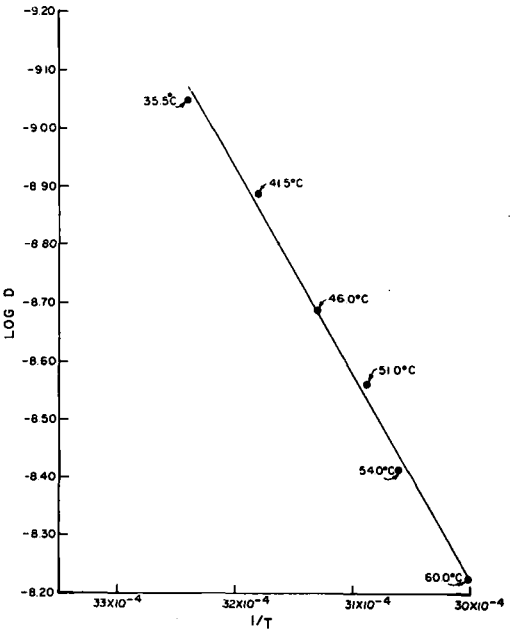

Fig. 18.—A plot of the log of D *vs.* the reciprocal of the absolute temperature for salicylic acid in nylon. [From Kapadia, A. J., Guess, W. L., and Autian, J., "Sorption and Diffusion Studies of Salicylic Acid in a Nylon Sheet," to be published.]

Effect of Other Ingredients in Plastics.—The discussion on sorption has dealt with a plastic which is considered composed of only the polymer, but it is known that many of the commercially available materials have various ingredients added for securing certain advantages. Sorption in these compounded plastics raised the question as to the possibility that the solute has interacted with one of the other ingredients, rather than the polymer. Reference may be made to an experiment conducted by Guess, Worrell, and Autian (85) on polyvinyl chloride used in medical practice. Benzalkonium chloride was found to be sorbed by the plastic to a significant degree, as may be noted by referring to Fig. 19. Even though no direct proof was obtained on the mechanism, it was believed that the benzalkonium chloride reacted with one of the other ingredients in the plastic formulation, since the structure of polyvinyl chloride would not suggest that this relatively nonpolar polymer would interact with a charged molecule to any appreciable extent.

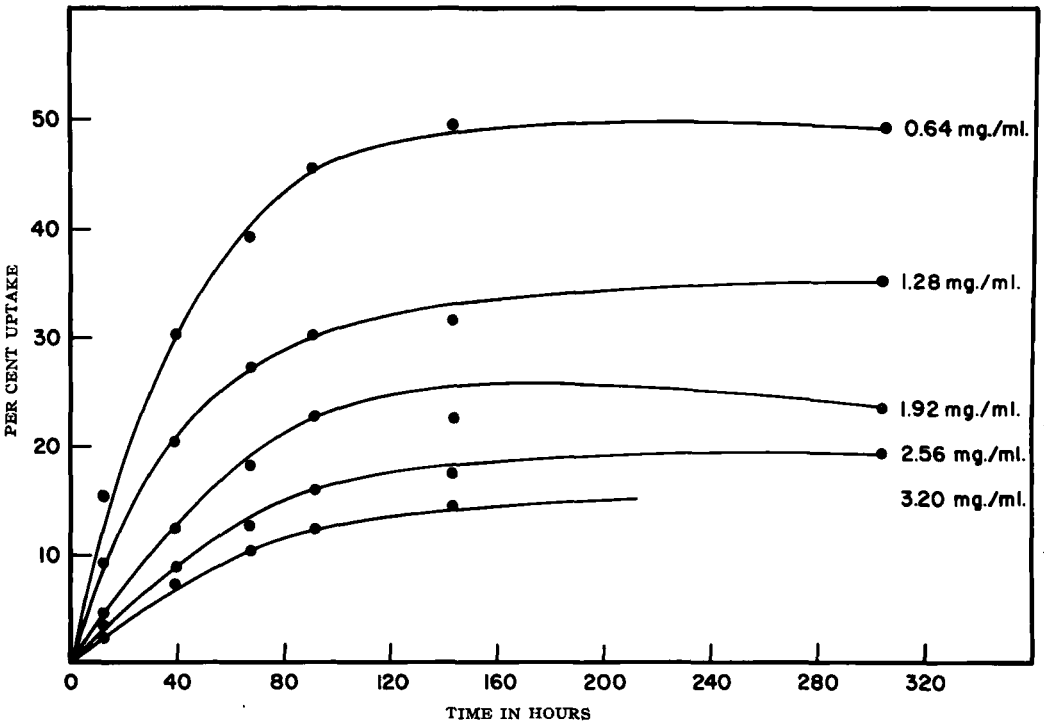

Fig. 19.—Effect of concentration on uptake of benzalkonium chloride by PVC. [From Guess, W. L., Worrell, L. F., and Autian, J., *Am. J. Hosp. Pharm.*, 19, 370(1962).]

Chemical Reactivity

The term *chemical reactivity* perhaps is not the best selection of a title for a discussion of plastics, since it appears that true chemical reactions between a polymer and a specific drug or similar agent are indeed far and few between. This does not, however, exclude the effect of oxygen under certain conditions of temperature and environment in reacting with a particular polymer, leading to eventual degradation of the plastic. Chemical reactivity here will refer to visible evidence that a particular plastic material is altered, or that a change has taken place in the physical characteristic of the solution. More of this type of information is available to the product development and packaging groups, rather than available in the literature and for this reason only a few selected instances will be cited which have been seen in the author's laboratory or have been brought to his attention.

Hartop has found that certain colorations in a plastic material are probably a result of one of the additives in the plastic, rather than due to the polymer itself (146). Since even the pure plastic materials usually have an antioxidant or a stabilizer there may always be the possibility for a stain developing on the plastic with certain ingredients in a drug product. This stain may also pass into the solution, adding a color or changing the color of the product. In a series of tests on various formulations of polyvinyl chloride with a number of parenteral products, several incompatibilities were observed. No further investigation as to the causative agent was pursued, but it was noted that in several cases, even though the exact material was employed, one drug product induced a color, while a second, having the same generic title, did not (2). The results of these studies do lend support to the contention that it will be quite difficult to predict performance in a plastic package until exacting testing programs are conducted on the whole drug product.

Since there are a variety of drugs and a variety of other ingredients used in pharmaceutical systems, one must expect occasional incompatibilities in which an agent or solvent has either etched or dissolved the plastic. This problem has already been touched upon under the section on leaching, but again, deserves some comments here.

Certain ingredients in parenteral products (as for example, paraldehyde, diethyl carbonate, benzyl alcohol, and benzaldehyde) will etch and dissolve polystyrene (147). Products of an oily nature may have a dissolving or softening effect upon polyethylene. The fluorinated hydrocarbons have been found to attack polystyrene, polyethylene, and most vinyl products. Brown has stated that the attack on plastics by the fluorinated hydrocarbons decreases as the number of fluorine atoms increase in the molecule (148).

When a chemical reaction occurs and it is easily evident, the product can be removed, thereby producing no harm to the patient or embarrassment to the manufacturer. What effect minor changes may have on the plastic or solution is a question which cannot be rightly answered. These changes usually take place after the product is out in the market or in the hands of the physician or patient. It will be interesting to see what results will turn up in the near future concerning plastic containers which have had long contact with drug systems under a variety of environmental conditions.

Alteration in the Physical Properties of Plastics

Such incidents as permeation, leaching, sorption, and chemical reactivity in regards to plastics will undoubtedly have an effect on one or more physical properties of the plastic. One of the more common observations with polyethylene bottles is the "bulging" or partial "collapse" of the container depending upon the direction of migration of a particular gas or vapor. Often this can be minimized by proper design of the container. A test which is frequently used to detect changes in physical characteristics is tensile strength measurements which may be defined as the force required to break by simple tension. As an example of the effect various liquids may have on the tensile strength of two plastic materials (polyethylene and polypropylene), Table IX is given. It will be noted that the linear polyethylene has the least ability to withstand these liquids. Surface-active agents, such as Igepal have a dramatic effect on polyethylene and, as has been stated in a previous portion of this review, Igepal is a recommended ingredient for testing of bottles. No real information is presently available as to the influence of drug systems on the tensile strength of plastics but it is suspected that certain combinations of ingredients might alter this physical property.

Temperature and changes of temperature can cause certain plastic objects to become distorted or, if the material contains plasticizers, the material may become brittle. Treatment of plastic items to various types of sterilization may alter

TABLE IX.—CHANGE IN TENSILE STRENGTH OF POLYETHYLENE AND POLYPROPYLENE ON LONG IMMERSION IN SELECTED LIQUIDS; 4 MONTHS OR MORE[a]

Liquid	Polyethylene		Polypropylene % Crystallinity	
	Low Density	Linear	63	56
Water	1.8	0	−1.1	−8.6
Isopropyl alcohol	1.1	−7.1	−7.1	−11.3
Primol D	−8.1	−78.0	10.1	−20.0
Silicone oil	−1.5	−11.0	13.8	−7.1
Methylethylketone	−5.2	−42.7	0	−6.0
Sodium hydroxide, 10%	−1.6	−14.0	3.2	−4.5
Common salt, 10%	2.6	−6.5	0	−9.1
Acetic acid, 10%	−8.1	9.8	13.2	−4.7
Dioctyl phthalate	−6.5	−2.3	5.9	−3.6
Linseed oil	−5.6	−60.5	1.1	−3.4
Corn oil	0	−50.5	16.0	−19.8
Methanol	2.2	2.5	2.3	−7.2
Igepal	−100	−100	9.8	−1.8

[a] Kresser, T. O. J., "Polypropylene," Reinhold Publishing Corp., New York, N. Y., 1960, p. 33.

the physical properties of the material to such a point that the device is no longer worthy of use. Preliminary reports on electron sterilization of plastic items have revealed that certain polypropylenes will take on a color while others will become brittle and break on touch (79). Ethylene oxide sterilization with certain Freons will disintegrate a number of plastic items which are used in hospitals (79).

Since pharmaceutical products are to be in contact with containers for long periods of time prediction of the behavior of the container over the history of the product will be extremely difficult without suitable testing programs. Even this approach may fail if a complete history of the container is not known. Plastic materials are also finding use in surgery where implants for the replacement of body organs or vessels are becoming more frequent. Exactly what effects the biological system will have on these plastic materials over long periods of time is a question which still needs to be answered.

SUMMARY

Plastics are no longer a curiosity item in the practice of pharmacy and medicine. Each year which passes finds more, and often better, uses for these polymeric materials. Since at the present time there are no real controls or standards for plastic items in the medical field, each group must select their plastic material with the greatest of care. This selection must be based upon thorough testing procedures to suit the needs of the product and the market. Problems can arise from improper use of plastics and public health responsibility requires that all in the chain of distributing the plastic article, be what it may, are morally liable for any adverse consequence.

This review was an attempt to acquaint those in the pharmaceutical and closely allied fields with the opportunities which plastics offer while at the same time exposing a number of present and possible future problems. If this paper contributes in some small way to eliminating or circumventing the problems which may be associated with plastics, while kindling greater interest in developing better uses for plastics in pharmacy and medicine, the author's efforts in composing the review will be justly rewarded.

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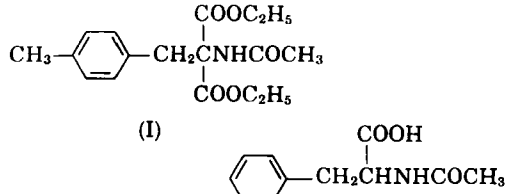
Research Articles

Methyl-Substituted Tyrosines and Related Compounds as Potential Anticancer Agents

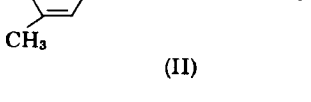
By EUGENE C. JORGENSEN and ROBERT A. WILEY†

3,5-Dimethyl-DL-tyrosine, 3-methyl-DL-tyrosine, 3-methyl-5-iodo-DL-tyrosine, as well as synthetic intermediates and derivatives of these, have been prepared for study of anticancer activity. Preliminary tests on two members of the series against experimental tumors in mice showed a slight inhibition of tumor growth.

THE SYNTHESIS and antitumor activity of a number of derivatives of phenylalanine, N-acetylphenylalanine, and diethyl α-acetamido-α-benzylmalonate have been reported (1, 2). The most active members of this series were diethyl α-acetamido-α-(4-methylbenzyl) malonate (I) and N-acetyl-3-methyl-DL-phenylalanine (II). Studies relative to the synthesis of analogs of thy-



(I)



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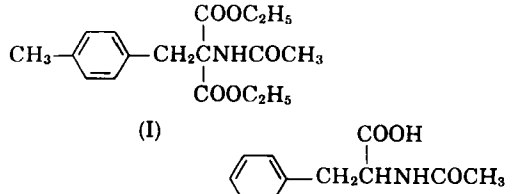
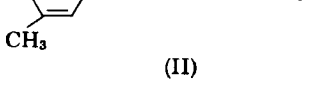
By EUGENE C. JORGENSEN and ROBERT A. WILEY†

3,5-Dimethyl-DL-tyrosine, 3-methyl-DL-tyrosine, 3-methyl-5-iodo-DL-tyrosine, as well as synthetic intermediates and derivatives of these, have been prepared for study of anticancer activity. Preliminary tests on two members of the series against experimental tumors in mice showed a slight inhibition of tumor growth.

THE SYNTHESIS and antitumor activity of a number of derivatives of phenylalanine, N-acetylphenylalanine, and diethyl α-acetamido-α-benzylmalonate have been reported (1, 2). The most active members of this series were diethyl α-acetamido-α-(4-methylbenzyl) malonate (I) and N-acetyl-3-methyl-DL-phenylalanine (II). Studies relative to the synthesis of analogs of thy-



(I)



(II)

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TABLE I.—SCREENING TEST FOR ANTICANCER ACTIVITY AGAINST ASCITES TUMORS IN MICE

Compound	Dose mg. per Kg. Body Weight	Gardner (ED) Lymphosarcoma TPCV,[a] %	Gardner (ED) Lymphosarcoma Survival,[b] days	Erhlich (E2) Carcinoma TPCV,[a] %	Erhlich (E2) Carcinoma Survival,[b] days	L4946 (C1) Leukemia TPCV,[a] %	L4946 (C1) Leukemia Survival,[b] days
V	500	126	+2.0	82	...	48	−1.5
VII	500	137	+2.0	65	...	77	−1.5

[a] TPCV = (Total packed cell volume treated/total packed cell volume controls). [b] Survival = (Median survival treated)-(median survival controls).

roxine involved the preparation of methyl-substituted derivatives of DL-tyrosine, and synthetic intermediates closely related to I. From these, a series of derivatives has been prepared for study as antitumor agents.

The chloromethylation procedure of Bielig (3) was adapted in the preparation of 3,5-dimethyl-4-methoxybenzyl chloride (III) and of 3-methyl-4-methoxybenzyl chloride (IV) from the corresponding methyl-substituted anisoles. 3,5-Dimethyl-4-methoxybenzyl chloride was condensed with ethyl acetamidocyanoacetate, and the resulting α-substituted cyanoacetic ester (V) converted into 3,5-dimethyl-DL-tyrosine (VI) by prolonged heating with hydriodic acid. Following the completion of this work, Dibbo, *et al.* (4), have reported a similar synthesis of 3,5-dimethyl-DL-tyrosine proceeding through the α-substituted diethyl acetamidomalonate (VII), which was also prepared in this study. This intermediate was used to prepare derivatives retaining the O-methyl group by selective hydrolysis to the acetamidomalonic acid (VIII), decarboxylation to the acetamido acid (IX), and further hydrolysis to form 3,5-dimethyl-4-methoxy-DL-phenylalanine (X).

3-Methyl-DL-tyrosine (XII) was obtained by the condensation of 3-methyl-4-methoxybenzyl chloride with diethyl acetamidomalonate, followed by hydrolysis of the ester (XI) with hydriodic acid. Iodination of XII in aqueous ethylamine yielded 3-methyl-5-iodo-DL-tyrosine (XIII).

The amino acids (VI, XII, XIII) were converted into their N-acetyl (XIV–XVI) and N-acetyl ethyl ester (XVII–XIX) derivatives.

Two of the intermediates, ethyl α-acetamido-α-cyano-β-(3,5-dimethyl-4-methoxyphenyl)-propionate (V) and diethyl α-acetamido-α-(3,5-dimethyl-4-methoxybenzyl)malonate (VII) were submitted for preliminary antitumor screening against lymphosarcoma, carcinoma, and leukemia tumors in mice[1] by the method of Costa, Blumenthal, and Greenberg (5). As shown in Table I, the compounds were inactive against the lymphosarcoma, but showed slight activity against the carcinoma (18 and 35% inhibition) and leukemia (52 and 23% inhibition). The remaining compounds herein reported will be submitted for antitumor screening.

EXPERIMENTAL[2]

3,5-Dimethyl-4-methoxybenzyl Chloride (III).—A mixture of 2,6-dimethylanisole (71 Gm., 0.5 mole), glacial acetic acid (500 ml.), and paraformaldehyde (20 Gm., 0.65 mole) was stirred and cooled in an ice bath until the temperature reached 10°. Concentrated hydrochloric acid (60 ml.) was added, and hydrogen chloride was passed into the stirred mixture for 11 hours. The temperature was held below 10° for the first 2 hours and at room temperature thereafter. The mixture was poured into 2 L. of ice water, and extracted with chloroform. The chloroform extract was washed successively with water, 1 *N* sodium bicarbonate solution, and water, and dried over anhydrous sodium sulfate. The chloroform was removed under reduced pressure, and the residual oil flash-distilled under nitrogen at reduced pressure. There was obtained 66.4 Gm. (68.6%) of an almost colorless liquid, n_D^{20} 1.5294, b.p. 124–128° (4 mm.) [Lit. (4), n_D^{20} 1.5375, b.p. 83–85° (1 mm.)].

Anal.—Calcd. for $C_{10}H_{13}ClO$: Cl, 19.25. Found: Cl, 18.89.

3-Methyl-4-methoxybenzyl Chloride (IV).—Prepared[3] from 2-methylanisole as described above, b.p. 125° (5 mm.), n_D^{20} 1.539.

Ethyl α-Acetamido-α-cyano-β-(3,5-dimethyl-4-methoxyphenyl)propionate (V).—To sodium (0.58 Gm., 0.025 mole) in absolute ethanol (10 ml.) was added in one portion ethyl acetamidocyanoacetate (4.25 Gm., 0.025 mole). 3,5-Dimethyl-4-methoxybenzyl chloride (III, 4.61 Gm., 0.025 mole) was added dropwise over 20 minutes to the stirred solution. The reaction mixture was heated under reflux for 1 hour, then poured into ice water. The yellow oil which separated solidified on standing to yield 6.99 Gm. (88%); recrystallization from methanol gave colorless plates, m.p. 139–140°.

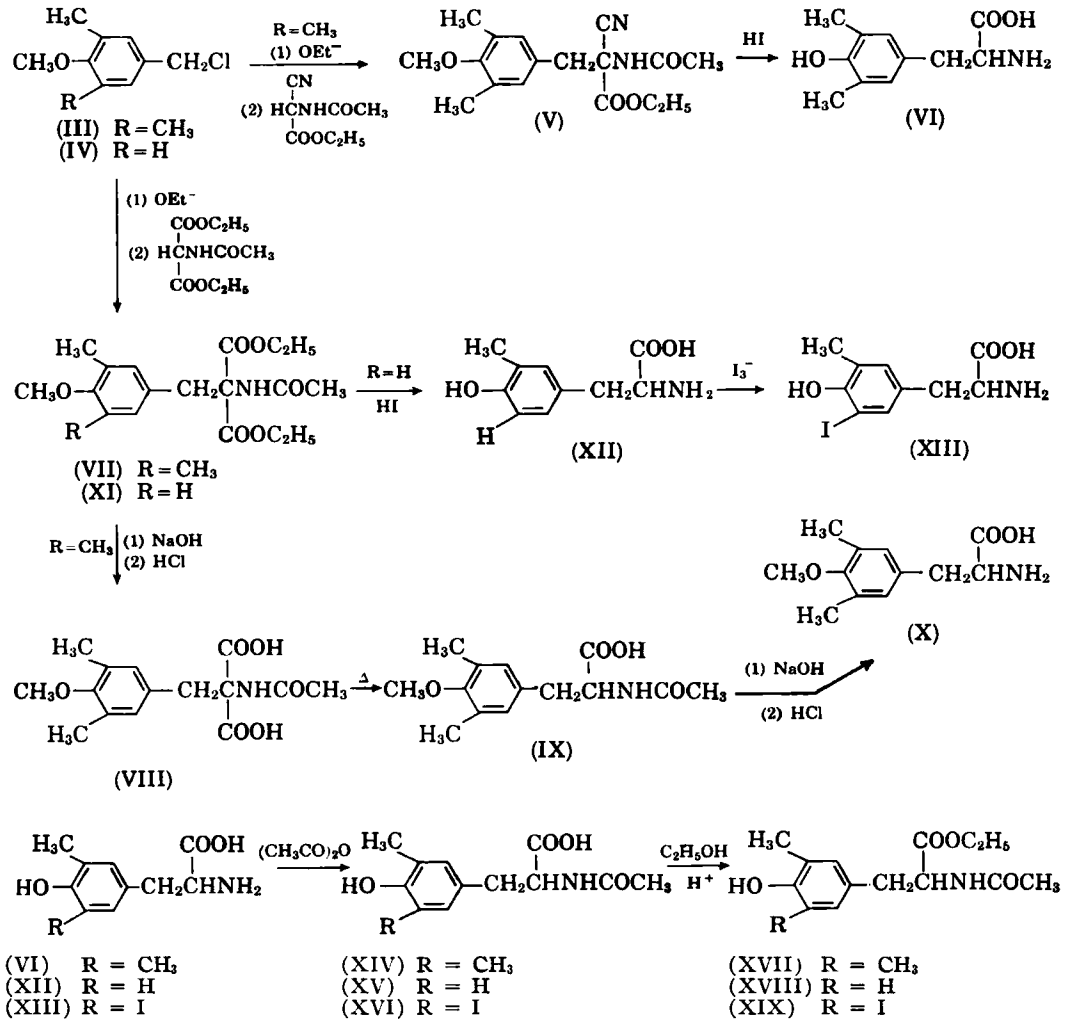
Anal.—Calcd. for $C_{17}H_{22}N_2O_4$: N, 8.81. Found: N, 8.66.

3,5-Dimethyl-DL-tyrosine (VI).—The preceding ester (V, 5.0 Gm., 15.4 mmoles) was heated under reflux for 4 hours with a mixture of glacial acetic acid (25 ml.) and 48% hydriodic acid (25 ml.). The reaction mixture was evaporated to dryness

[1] The authors are indebted to Dr. D. M. Greenberg for the biological results.

[2] Melting points obtained on a Fisher-Johns melting point apparatus and are uncorrected. Microanalyses by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley.

[3] Prepared by Mr. Menlo Kawakami, to whom we express our gratitude.



in vacuo, and the light pink residue dissolved in hot water (5 ml.). Concentrated sodium acetate solution was added to pH 5; after overnight refrigeration, 3.03 Gm. (92%) of yellowish powder was collected. An analytical sample was prepared by iso-electric precipitation at pH 5, m.p. 240–243° (decompn.) [Lit. (4), 242–248° (decompn.)].

Anal.—Calcd. for $C_{11}H_{15}NO_3$: C, 63.16; H, 7.23. Found: C, 62.88; H, 7.13.

Ethyl α-Acetamido-α-(3,5-dimethyl-4-methoxybenzyl)malonate (VII).—To sodium (1.15 Gm., 0.05 mole) dissolved in absolute ethanol (125 ml.) was added in one portion ethyl acetamidomalonate (10.85 Gm., 0.05 mole). 3,5-Dimethyl-4-methoxybenzyl chloride (III, 9.22 Gm., 0.05 mole) was added dropwise over 20 minutes to the stirred solution. The reaction mixture was heated under reflux for 1 hour, then poured into ice water. The oil which separated soon solidified, affording 11.6 Gm. (64%) of colorless prisms. Recrystallized from hexane it had m.p. 94–95° [Lit. (4), 97–99°].

Anal.—Calcd. for $C_{19}H_{27}NO_6$: C, 62.44; H, 7.45. Found: C, 62.59; H, 7.39.

Ethyl α-Acetamido-α-(3-methyl-4-methoxybenzyl)malonate (XI).—Prepared[3] from 3-methyl-4-methoxybenzyl chloride (IV) as described above in 83.5% yield, m.p. 106–107°. An unstable polymorphic form melting at 113° was also observed, which reverted to the lower melting, more stable form on standing.

Anal.—Calcd. for $C_{18}H_{25}NO_6$: C, 61.51; H, 7.17. Found: C, 61.77; H, 6.99.

α - Acetamido - α - (3,5 - dimethyl - 4 - methoxybenzyl)malonic Acid (VIII).—The malonate ester (VII, 2.0 Gm., 5.47 mmoles) was dissolved in a solution of sodium hydroxide (5 Gm.) in 50% ethanol (50 ml.) and stirred at room temperature for 24 hours. Most of the alcohol was evaporated *in vacuo*, and the unreacted ester removed from the aqueous residue by extraction with chloroform. The solution was cooled to 10° and acidified with concentrated hydrochloric acid, whereupon 1.0 Gm. (59%) of white precipitate separated. This was crystallized from acetone-water, and from acetone-hexane, m.p. 143–144°.

Anal.—Calcd. for $C_{15}H_{19}NO_6$: C, 58.28; H, 6.19. Found: C, 58.49; H, 6.26.

N - Acetyl - 3,5-dimethyl - 4 - methoxy - DL-

phenylalanine (IX).—The preceding acetamido acid (VIII, 0.8 Gm., 2.6 mmoles) was heated at a bath temperature of 180° for 30 minutes. The resulting yellow oil was taken up in chloroform, extracted with 2% sodium hydroxide, the aqueous extract acidified, and extracted with ether. The ether solution was dried over anhydrous sodium sulfate, filtered, and allowed to evaporate in the air, affording 0.3 Gm. (44%) of colorless prisms. Crystallized from ethanol-water, it had m.p. 151.5–152°.

Anal.—Calcd. for $C_{14}H_{19}NO_4$: C, 63.39; H, 7.22. Found: C, 63.31; H, 7.20.

3,5-Dimethyl-4-methoxy-DL-phenylalanine (X).—The preceding N-acetyl derivative (IX, 3.4 Gm., 12.8 mmoles) was heated under reflux with 100 ml. of 10% aqueous sodium hydroxide for 24 hours. Acidification with concentrated hydrochloric acid resulted in a white inorganic precipitate which was removed by filtration, and the filtrate evaporated to dryness *in vacuo*. The residue was extracted with 250 ml. of boiling absolute ethanol which, on evaporation, yielded 0.7 Gm. (25%), m.p. 250° (decompn.). Purification was effected by isoelectric precipitation at pH 5.

Anal.—Calcd. for $C_{12}H_{17}NO_3$: C, 64.50; H, 7.67. Found: C, 64.22; H, 7.76.

3-Methyl-DL-tyrosine (XII).—The acetamidomalonic ester (XI, 25 Gm., 0.071 mole), 47% hydriodic acid (100 ml.), and glacial acetic acid (100 ml.) were heated under reflux for 7 hours. Additional hydriodic acid (60 ml.) was added and heating was continued overnight. The acids were removed by distillation *in vacuo*, the residue taken up in water, and the pH of the solution adjusted to 5 with 40% aqueous sodium hydroxide, affording 21.6 Gm. of white solid. One isoelectric precipitation at pH 5 yielded 12 Gm. (86%) of white amorphous powder, m.p. 285° (decompn.) [Lit. (6), 276°].

3-Methyl-5-iodo-DL-tyrosine (XIII).—To a well-stirred solution of 3-methyl-DL-tyrosine (XII, 10 Gm., 0.051 mole) in 33% aqueous ethylamine (100 ml.), a solution of iodine (15.5 Gm., 0.061 mole) and potassium iodide (20 Gm.) in water (100 ml.) was added dropwise at room temperature. After the addition was complete, stirring was continued for 30 minutes, excess iodine was reduced with aqueous sodium bisulfite, and the pH of the solution was adjusted to 5 with acetic acid. The resulting precipitate was removed by filtration and purified by isoelectric precipitation at pH 5, yielding 12.2 Gm. (74.5%), m.p. 213–214° (decompn.). The compound was not obtained analytically pure.

N-Acetyl-3,5-dimethyl-DL-tyrosine (XIV).—To 3,5-Dimethyl-DL-tyrosine (VI, 1.5 Gm., 7.18 mmoles) dissolved in 2 *N* sodium hydroxide (31 ml.) and maintained at 5–10°, acetic anhydride (3.45 ml.) was added over a period of 1.5 hours. After standing overnight at room temperature, the solution was cooled to 10° and a solution of sodium hydroxide (1.49 Gm.) in water (3.8 ml.) was added, followed by ethanol (18 ml.), and concentrated hydrochloric acid to pH 1.5. Upon partial evaporation, 1.42 Gm. of brownish crystals was deposited. Crystallization from ethanol-water yielded 1.0 Gm. (55%) of colorless crystals, m.p. 182–183° [Lit. (4), 183–185°].

Anal.—Calcd. for $C_{12}H_{17}NO_4$: C, 62.12; H, 6.82. Found: C, 62.51; H, 7.19.

N-Acetyl-3-methyl-DL-tyrosine (XV).—3-Methyl-DL-tyrosine (XII) was acetylated as described above. The resulting oil resisted crystallization, but was identified by conversion to the N-acetyl ethyl ester (XVIII).

N-Acetyl-3-methyl-5-iodo-DL-tyrosine (XVI).—The N-acetyl derivative was prepared in 80% yield from 3-methyl-5-iodo-DL-tyrosine (XIII) as previously described, m.p. 180–181°.

Anal.—Calcd. for $C_{12}H_{14}INO_4$: C, 39.68; H, 3.89. Found: C, 39.87; H, 4.03.

N-Acetyl-3,5-dimethyl-DL-tyrosine Ethyl Ester (XVII).—N-Acetyl-3,5-dimethyl-DL-tyrosine (XIV, 4.7 Gm., 18.7 mmoles), *p*-toluenesulfonic acid (0.6 Gm.), absolute ethanol (6 ml.), and chloroform (60 ml.) were heated together under reflux for 8 hours, during which the azeotropic mixture of water, ethanol, and chloroform was removed by distillation. Additional chloroform was added as needed to maintain a minimum volume of 60 ml. After 4 hours an additional 5 ml. of absolute ethanol was added. The reaction mixture was washed with 5% aqueous sodium bicarbonate and with water, dried over anhydrous calcium chloride, and the chloroform removed by distillation under reduced pressure. The residual brown oil solidified upon standing, and was crystallized from ethanol-water and from ethyl acetate-hexane, yielding 3.8 Gm. (72%), m.p. 96–97° [Lit. (4), 101–102°].

Anal.—Calcd. for $C_{15}H_{21}NO_4$: C, 64.48; H, 7.58. Found: C, 64.20; H, 7.33.

N-Acetyl-3-methyl-DL-tyrosine Ethyl Ester (XVIII).—The oily N-acetyl derivative (XV) was esterified as described above, yielding a crystalline compound, m.p. 133–134°. The overall yield from 3-methyl-DL-tyrosine was 56%.

Anal.—Calcd. for $C_{14}H_{19}NO_4$: C, 63.38; H, 7.22. Found: C, 63.57; H, 7.11.

N-Acetyl-3-methyl-5-iodo-DL-tyrosine Ethyl Ester (XIX).—The N-acetyl derivative (XVI) was esterified as described above, yielding (66%) a crystalline compound, m.p. 126–127°.

Anal.—Calcd. for $C_{14}H_{18}INO_4$: C, 42.98; H, 4.64. Found: C, 43.03; H, 4.50.

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Studies on the Mechanism of Action of Phenolic Disinfectants II

Patterns of Release of Radioactivity from *Escherichia coli* Labeled by Growth on Various Compounds

By JOSEPH JUDIS

Previous studies on the leakage of radioactivity from glutamate-C^{14} labeled *Escherichia coli* cells in the presence of phenolic disinfectants were extended to involve cells labeled by growth on glucose-U-C^{14}, acetate-C^{14}, adenine-C^{14}, sulfate-S^{35}, and phosphate-P^{32}. In general, the phenolic disinfectants considered caused varying amounts of radioactivity to be released from the labeled cells. The phenol derivatives did not cause leakage of significant amounts of radioactivity from heat-treated cells, and cells exposed to *n*-butanol (which breaks the osmotic barrier) did release additional radioactivity when treated with *p*-chloro-*m*-xylenol or phenol, but the total counts released under the experimental conditions did not exceed that with further butanol treatment. Cells labeled with P^{32}, once treated with *p*-chloro-*m*-xylenol, continued to release radioactivity in saline buffer but glucose-C^{14}-labeled cells did not. The amount of radioactivity released from variously labeled cells in the presence of phenol, *p*-chloro-*m*-xylenol, or *n*-butanol was affected by temperature.

In previous studies (1), it was shown that *Escherichia coli*, when labeled by incubation with glutamate-C^{14}, would lose radioactivity when exposed to phenolic disinfectants. This loss of radioactivity was proportional to the concentration of the phenol derivative and was caused by all of the phenols tested. It was assumed that the radioactivity lost could be due to extrusion of cell contents through a damaged cell membrane. This hypothesis was further investigated by studying the release of radioactivity in the presence of phenol derivatives from *E. coli* labeled by growth on a number of radioactive compounds.

METHODS AND MATERIALS

Materials.—Only reagent grade chemicals were used and biochemicals were obtained from Nutritional Biochemicals Corp. or the California Corp. for Biochemical Research. The radioactive compounds were obtained either from the California Corp. for Biochemical Research, New England Nuclear Corp., or Abbott Laboratories. The labeled organic compounds had the following specific activities (mc./mmole): glucose-U-C^{14}, 4; sodium acetate-1,2-C^{14}, 10.5; adenine-8-C^{14}, 14.4; sodium glutamate-3,4-C^{14}, 2.3. Phosphorus^{32} was purchased as NaH_2PO_4 in solution with an activity of 10 μc./5 ml., and sodium sulfate-S^{35} was obtained with an activity of 50 μc./5 ml. The organic compounds were dissolved in distilled water to give a concentration of 2 μc./ml. A number of the phenol derivatives were a gift of the Ottawa Chemical Co., Toledo, Ohio.

Labeling of Bacteria.—The procedure for labeling *Escherichia coli* ATCC 11229 (the strain used throughout) with glutamate-C^{14} has been previously described (1). The synthetic medium (C) of Roberts, *et al.* (2), was used for growth as previously. Glucose-C^{14}, sodium sulfate-S^{35}, or sodium phosphate-P^{32} were added to the medium at the time of inoculation. In the case of P^{32}, the total phosphate content of the medium was reduced to one-fourth of the usual to permit a greater uptake of P^{32}. Approximately 1–2 μc. of glucose-C^{14} or sodium phosphate-P^{32}, or 10–20 μc. of sodium sulfate-S^{35} were added to 200 ml. of medium in a 500-ml. Erlenmeyer flask. The culture flasks were incubated on a Burrell wrist action shaker for 24 hours at 37° and harvested by centrifugation, followed by three washes with cold saline to remove any medium constituents. In the case of adenine-C^{14} and sodium acetate-C^{14}, normally two flasks (200 ml. of medium per flask) were grown for 24 hours, as above, without the addition of the labeled compounds. The cells were harvested by centrifugation and resuspended in one flask of fresh medium containing 2 μc. of radioactive acetate or adenine. The flask was shaken for an additional 3 hours at 37°, after which the cells were harvested and washed as above. During growth on labeled organic compounds, the flasks were attached to a U-tube containing soda lime to trap $C^{14}O_2$.

The washed, labeled cells were suspended in saline to give a concentration of 4×10^{10} cells/ml., as determined by turbidity readings previously calibrated with plate counts.

Measurement of Release of Radioactivity.—Basically, the previously described procedures (1) were used throughout. Phenol derivatives (except phenol) were dissolved in 25% v/v ethanol. Reaction mixtures contained, in a total volume of 3.0 ml., 2.4×10^{10} cells, pH 7.2 phosphate buffer in a final concentration of 0.02 *M*, the phenol derivative in a volume of 0.5 ml. (or made up to 0.5 ml. with 25% v/v ethanol) and enough saline

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to bring to volume. The reaction mixtures were incubated at 22 ± 2° for a total of 10 minutes and centrifuged for 20 minutes. Aliquots of the supernatant and the cells were dried on 1¼ inch, concentric ring stainless steel planchets, and radioactivity was assayed in a Nuclear-Chicago thin window gas flow counter.

In the experiments in which the amount of radioactivity released was measured at time intervals, a number of replicate reaction mixtures were prepared and removed at the indicated times for centrifugation (5 minutes at 14,000 r.p.m.).

RESULTS AND DISCUSSION

A bactericidal substance may act by a number of possible mechanisms which could be roughly categorized as either metabolic or physical-chemical (structural damage). Undoubtedly, a given substance may combine these mechanisms. In the case of phenolic disinfectants, there has been relatively little work on metabolic effects. Recently published data (1, 3–10) stress the effects of phenol derivatives on permeability mechanisms and suggest that damage to the latter are responsible ultimately for their killing effect. The results shown in Table I would tend to indicate that in the concentrations used, phenol derivatives do cause leakage of radioactivity from *Escherichia coli* labeled by growth on a number of compounds. The highest concentration used of each phenol derivative was adequate to cause at least 99% killing or more under the experimental conditions (1) except 2,4,6-trichlorophenol which caused 32% killing and 2,4-dichloro-*m*-xylenol, 97% killing.

Roberts, *et al.* (2), labeled *E. coli* by growth on a number of radioactive compounds (such as glucose, acetate, purines, sulfate-S^{35}, and phosphate-P^{32}) and fractionated the cells to determine into which type of compounds the label was incorporated. For example, inorganic sulfate was incorporated primarily into protein; phosphate into nucleic acids primarily but also phospholipids and metabolic intermediates; acetate equally into lipids and protein; and glucose, primarily into protein but also nucleic acids, lipids, and some metabolic intermediates. It was hoped that if phenol derivatives would cause release of radioactivity from cells labeled by growth on specific compounds to a much greater degree than when labeled with other compounds, some insight might be obtained as to what the substrate, in general terms, might be for phenols.

The data in Table I would seem to suggest that phenol derivatives cause release of the highest per cent of radioactivity of cells labeled by growth on adenine-C^{14} or phosphate-P^{35}, both of which are reported as being incorporated primarily into nucleic acids. Both DNA and RNA have been detected in isolated membranes (11, 12) although nucleic acids, nucleotides, and other phosphorus compounds could also originate from within the cell. Joswick (10) has reported the release of 260 mμ-absorbing substances (purines and pyrimidines) from bacteria in the presence of hexachlorophene, and the release of 260 mμ-absorbing substances is generally taken as an index of damage to the mechanisms which control permeability of the cell.

The substantial amounts of radioactivity released from cells labeled by growth on sulfate-S^{35}, which is incorporated primarily into protein, could also originate in the cell membrane which is known to contain considerable protein (13). Radioactivity from acetate-C^{14} cells, which is reported to be incorporated about equally into

TABLE I.—RELEASE OF RADIOACTIVITY FROM LABELED *Escherichia coli*[a] IN THE PRESENCE OF VARIOUS PHENOLIC DISINFECTANTS

Treatment		Net[b] Per Cent of Radioactivity Released from Cells Labeled by Growth on					
		Glucose-C^{14}	Acetate-C^{14}	Adenine-C^{14}	$Na_2S^{35}O_4$	$NaH_2P^{32}O_4$	Glutamate-C^{14}
p-Chloro-*m*-xylenol	167 mcg./ml.	1.3	1.4	2.6	3.8	3.6	...
	250 mcg./ml.	2.7	3.1	5.5	5.8	3.9	...
	333 mcg./ml.	3.0	3.7	8.7	6.2	9.1	39.2
Phenol	3.3 mg./ml.	...	0.8	0.5	...	3.8	...
	8.3 mg./ml.	...	3.5	3.1	4.0	10.1	...
	12.5 mg./ml.	3.7	6.1	6.3	5.0	12.7	54.3
p-Chloro-*m*-cresol	333 mcg./ml.	1.4	1.0	2.4	0.5	8.2	24.3
	666 mcg./ml.	3.2	2.8	7.4	4.5	11.1	37.6
p-Chloro-*o*-cresol	333 mcg./ml.	1.2	0.8	2.2	0.3	6.9	11.8
	666 mcg./ml.	2.6	4	5.1	4.4	12.3	26.2
2,4-Dichloro-*m*-xylenol	41.7 mcg./ml.	0.3	...	...	0.2	...	15.8
	83 mcg./ml.	1.5	...	...	3.1	...	...
2,4-Dichlorophenol	333 mcg./ml.	2.0	0.8	...	0.4	...	8.9
	666 mcg./ml.	3.3	1.1	...	4.1	...	29.0
2,4,6-Trichlorophenol	167 mcg./ml.	0.3	...	...	0	...	...
	333 mcg./ml.	0.6	...	...	0.1	...	14.0
Hyamine 3500,[c] 1:500							50.0
1:1000		14.7	24.8	8.2	7.4	...	...
n-Butanol, 5% v/v		8.7	13.9	10.4	1.6	7.2	...
Heated in boiling water bath for 5 min.		8.4	9.1	18.3	14.2	17.2	...
Saline-buffer control		1.9	1.5	2.5	0.9	5.7	4.9
Ethanol (4.2% v/v)-buffer control		2.2	1.8	2.9	1.1	5.6	21.9

[a] The cell concentrations in the reaction mixtures were 8 × 10^9 cells/ml., with the exception of the cells labeled with glutamate-C^{14} which was 3.8 × 10^9 cells/ml. The labeled cells had approximately the following activities (c.p.m./10^9 cells): glucose-C^{14}, 300; acetate-C^{14}, 300; adenine-C^{14}, 2600; sulfate-S^{35}, 1500; phosphate-P^{32}, 40 to 186; glutamate-C^{14}, 520. [b] The radioactivity released in the appropriate control (ethanol-buffer for phenolic disinfectants except phenol and saline-buffer for others) was subtracted to obtain the net leakage. [c] Alkyl (C_{12}–C_{16}) dimethyl benzyl ammonium chloride, Rohm and Haas Co., Philadelphia, Pa.

lipids and proteins (2) of *E. coli*, is also released. The greatest release was found with glutamate-C^{14}-labeled cells and, although it is not clear as to what the glutamate is incorporated into, it is rapidly incorporated into nondiffusible compounds (2) and would not be simply adsorbed to the cells. While it cannot be said that the data in Table I indicate damage to the cell membrane as the basis for the lethal action of phenols, they are compatible with this hypothesis.

It would seem that the source of the radioactivity released from labeled cells must be due either to weakening or destruction of the permeability barrier of the cell, or "uncoupling" of protoplasmic constituents, or a combination of the two. If phenolic disinfectants act by affecting the permeability barriers, they should not cause release of additional radioactivity from cells whose permeability barrier has been destroyed. This can be accomplished by heating the cells, which causes a rapid release of the cellular pool and a certain amount of cytoplasmic degradation (14), or treatment with *n*-butanol, which breaks the osmotic barrier (15).

In Tables II, III, and IV, it can be seen that neither phenol, *p*-chloro-*m*-xylenol (PCMX), or butanol caused significant additional release of radioactivity from heated cells, thus indicating little or no additional cytoplasmic degradation. These results are in agreement with Beckett, *et al.* (14), who studied the release of 260 mμ-absorbing exudate in heat-treated *E. coli*, followed by hexylresorcinol. When labeled cells were first treated with butanol, and then retreated with phenol, *p*-chloro-*m*-xylenol, or butanol, the total counts released never exceeded that obtained with butanol, although the rate of release varied. Beckett, *et al.* (14), concluded from their data that hexylresorcinol caused extensive intracellular uncoupling because much more exudate was released from *E. coli* after preliminary treatment with butanol (which broke the osmotic barrier) than without preliminary treatment of the cells. The data in Tables II, III, and IV seem to be more compatible with the contention that the phenol derivatives considered, like butanol, act by breaking the permeability barrier.

It is interesting to speculate as to whether the alleged damage caused to the cell membrane is quantitatively related to the amount of phenol derivative to which the cell is exposed or whether the effect is such that once small damages are caused, they rapidly enlarge and leakage proceeds continuously. Experimentally, this was approached by treating cells with *p*-chloro-*m*-xylenol, and then exposing these cells to either saline-buffer, butanol, or additional treatments with *p*-chloro-*m*-xylenol. The results obtained are given in Tables V and VI. It would seem that phosphate-P^{32}-labeled cells continued to release some radioactivity once treated with *p*-chloro-*m*-xylenol, and additional exposure to the latter did not markedly affect the release of

TABLE II.—ABILITY OF PHENOL AND *p*-CHLORO-*m*-XYLENOL TO CAUSE RELEASE OF RADIOACTIVITY FROM HEATED OR *n*-BUTANOL-TREATED *Escherichia coli*[a] LABELED BY GROWTH ON SULFATE-S^{35}

Original Treatment of Cells[a]	Extraction Medium	Original Treatment	Radioactivity Released, c.p.m. Extraction No. 1	2	3	Total	Radioactivity Left in Cells, c.p.m.
Heated in boiling water bath for 5 min.	Phenol (12.5 mg./ml.)	4155	126	63	45	4389	15,292
	PCMX (333 mcg./ml.)	4329	105	57	36	4527	15,292
	n-Butanol (5% v/v)	4173	153	66	66	4458	14,683
	Ethanol (4.2% v/v)-buffer	4233	141	27	33	4434	15,042
	Saline-buffer	4188	146	45	33	4412	14,556
n-Butanol (5% v/v)	Phenol (12.5 mg./ml.)	885	1773	966	444	4068	16,983
	PCMX (333 mcg./ml.)	1272	966	765	720	3723	18,144
	n-Butanol (5% v/v)	1012	1257	822	996	4087	17,795
	Ethanol (4.2% v/v)-buffer	1215	852	438	396	2901	18,163
	Saline-buffer	1737	1215	597	423	3972	17,508

[a] The cells had an activity of 1182 c.p.m./10^9 cells.

TABLE III.—ABILITY OF PHENOL AND *p*-CHLORO-*m*-XYLENOL TO CAUSE RELEASE OF RADIOACTIVITY FROM HEATED OR *n*-BUTANOL-TREATED *Escherichia coli*[a] CELLS LABELED BY GROWTH ON ADENINE-C^{14}

Original Treatment of Cells	Extraction Medium	Original Treatment	Radioactivity Released, c.p.m. Extraction No. 1	2	3	Total	Radioactivity Left in Cells, c.p.m.
Heated in boiling water bath for 5 min.	Phenol (12.5 mg./ml.)	16,708	435	314	165	17,622	20,491
	PCMX (333 mcg./ml.)	17,737	434	185	195	18,551	20,491
	n-Butanol (5% v/v)	16,523	434	206	178	17,341	19,675
	Ethanol (4.2% v/v)-buffer	15,686	435	100	101	16,322	20,156
	Saline-buffer	17,515	399	103	147	18,163	19,505
n-Butanol (5% v/v)	Phenol (12.5 mg./ml.)	8,473	3437	8849	374	21,133	22,757
	PCMX (333 mcg./ml.)	8,599	2023	3226	2,285	16,133	24,313
	n-Butanol (5% v/v)	8,422	1971	4299	10,235	24,928	23,845
	Ethanol (4.2% v/v)-buffer	8,086	1742	1461	3,016	14,305	24,338
	Saline-buffer	7,694	1964	4515	6,101	20,279	23,461

[a] The cells had an activity of 2049 c.p.m./10^9 cells.

radioactivity. However, glucose-C^{14}-labeled, *p*-chloro-*m*-xylenol-treated cells did not release additional radioactivity when extracted with saline-buffer or ethanol-buffer. Also additional treatment with *p*-chloro-*m*-xylenol did not cause release of much additional radioactivity.

It is generally assumed that the antimicrobial activity of phenol increases with temperature (16, 17) and one would presume that phenomena associated with killing of bacteria by phenol derivatives should respond similarly. Table VII presents the per cent of radioactivity released from variously labeled cells in the presence of phenol, *p*-chloro-*m*-xylenol, and *n*-butanol at three temperatures. In general, an increase in temperature caused an increase in release of radioactivity. The only exception occurred with sulfate-S^{35}-labeled cells. It would seem that the latter observation coupled with the low release of radioactivity caused in sulfate-S^{35}-labeled cells by butanol would suggest an origin of the radioactivity which might be different from that obtained from cells labeled in other ways. Perhaps the fact that phenol derivatives cause the release of more counts from sulfate-S^{35}-labeled cells than butanol may indicate that some of the counts are coming from surface layers rather than low molecular weight compounds which would be released simply by breaking the osmotic barrier. Joswick (10) described a biphasic release of cell exudate caused by hexachlorophene which is also known to occur with other surface-active detergents (18) and polymyxin (19). He considers the leakage at 0° to be related to membrane damage, and at higher temperatures to be related to autolysis.

TABLE IV.—ABILITY OF PHENOL AND *p*-CHLORO-*m*-XYLENOL TO CAUSE RELEASE OF RADIOACTIVITY FROM HEATED OR *n*-BUTANOL-TREATED *Escherichia coli* CELLS[a] LABELED BY GROWTH ON GLUCOSE-C^{14}

Original Treatment of Cells	Extraction Medium	Original Treatment	Radioactivity Released, c.p.m. Extraction No. 1	2	3	Total	Radioactivity Left in Cells c.p.m.
Heated in boiling water bath for 5 min.	Phenol (12.5 mg./ml.)	877	66	31	71	1045	6512
	PCMX (333 mcg./ml.)	856	46	22	17	940	5773
	n-Butanol (5% v/v)	785	53	40	31	908	5372
	Ethanol (4.2% v/v)-buffer	899	40	23	25	987	5597
	Saline-buffer	936	59	18	30	1043	7170
n-Butanol (5% v/v)	Phenol (12.5 mg./ml.)	623	409	189	37	1258	6027
	PCMX (333 mcg./ml.)	634	352	270	92	1348	6050
	n-Butanol (5% v/v)	491	509	282	79	1361	6378
	Ethanol (4.2% v/v)-buffer	572	292	197	58	1118	7310
	Saline-buffer	555	236	253	47	1091	6490

[a] The cells had an activity of 390 c.p.m./10^9 cells.

TABLE V.—EFFECT OF RE-EXPOSURE TO *p*-CHLORO-*m*-XYLENOL ON RELEASE OF RADIOACTIVITY FROM *Escherichia coli*[a] LABELED BY GROWTH ON PHOSPHATE-P^{32}

Original Treatment of Cells	Extraction Medium	Original Treatment	Radioactivity Released, c.p.m. Extracton No. 1	2	3	Total	Radioactivity Left in Cells c.p.m.
PCMX, 333 mcg./ml.	Saline-buffer	294	424	293	167	1178	1183
	Ethanol (4.2% v/v)-buffer	276	276	179	183	914	1306
	PCMX (333 mcg./ml.)	258	385	349	140	1132	1183
	n-Butanol (5% v/v)	274	340	431	204	1249	1034
Ethanol (4.2% v/v)-control	Ethanol (4.2% v/v)-buffer	166	122	69	47	403	1794

[a] The cells had an activity of 117 c.p.m./10^9 cells.

TABLE VI.—ABILITY OF *p*-CHLORO-*m*-XYLENOL AND *n*-BUTANOL TO CAUSE RELEASE OF RADIOACTIVITY FROM *p*-CHLORO-*m*-XYLENOL-TREATED *Escherichia coli*[a] LABELED BY GROWTH ON GLUCOSE-C^{14}

Original Treatment of Cells	Extraction Medium	Original Treatment	Radioactivity Released, c.p.m. Extraction No. 1	2	3	Total	Radioactivity Left in Cells c.p.m.
PCMX, 333 mcg./ml.	Saline-buffer	423	104	46	46	619	7818
	Ethanol (4.2% v/v)-buffer	405	85	53	65	608	8046
	PCMX (333 mcg./ml.)	425	140	131	162	858	7499
	n-Butanol (5% v/v)	436	224	75	65	800	7118
Ethanol (4.2% v/v)-control	Saline-buffer	225	84	36	42	387	7866
	Ethanol (4.2% v/v)-buffer	204	84	51	64	403	7953
	PCMX (333 mcg./ml.)	243	290	110	141	784	7690
	n-Butanol (5% v/v)	207	332	107	107	753	7370

[a] The cells had an activity of 368 c.p.m./10^9 cells.

However, in the experiment for which results are presented in Table VII, insufficient time would have elapsed for significant autolysis.

Figures 1 and 2 indicate the rates of release of radioactivity from either phosphate-P^{32} or sulfate-S^{35}-labeled cells in constant contact with *p*-chloro-*m*-xylenol. The difference in the shapes of these two curves also suggest the mechanism of release and/or the origin or identity of the radioactive compounds released may be different. The control curves indicate a greater tendency for leakage after 1 to 2 hours, of P^{32}-labeled compounds than of S^{35}-labeled compounds.

In general, the observations presented above and others would suggest that phenols exert their lethal action by physical damage to the permeability barriers. It is quite reasonable to expect that a relatively minor amount of physical damage, such as alteration in permeability without lysis, could be lethal. For example, Strauss (20) reported the formation of a lethal permeability defect in *E. coli* by a mutagenic alkylating agent, ethyl sulfate. On the other hand, Nathan (21) described a permeability change in *Lactobacillus plantarum* caused by chlorpromazine which was nonlethal. It must still be recognized that evidence regarding permeability damage and lethal effects of phenols is circumstantial and, as has been shown by Stedman, *et al.* (22), and Kravitz, *et al.* (23), the total effect of a germicide (a quaternary ammonium compound in their studies) may involve, to different extents, leakage of cellular materials and inhibition of energy yielding reactions. The contribution of each of these effects may depend upon a number of conditions, and varying the latter will vary the influence of a given effect. There are a number of additional approaches to examining the mechanism of the lethal effects of phenolic disinfectants, such as determining the cytological site of uptake of these compounds. If the cell membrane is the site of action of phenolic disinfectants, one should be able to assume that the latter would be bound to the membrane and concentrate there, at least until the integrity of the latter is destroyed and the germicide penetrates into the cell. It would be useful to determine the relative distribution of labeled phenolic disinfectants in the usual fractions obtained in cell fractionation, or even better, among the basic structural fractions, such as the cell wall, cell membrane, and cytoplasm. Beckett, *et al.* (4, 5), have already shown that cell walls from *E. coli* do not bind hexylresorcinol although the whole cell does.

More precise knowledge of the identity of the substances which leak from the cell would be useful for determining to what extent phenol derivatives

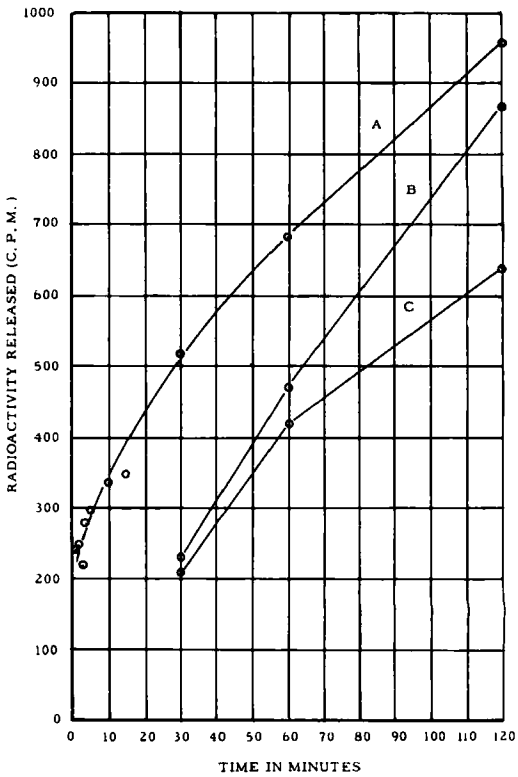

Fig. 1.—Release of radioactivity from *Escherichia coli* labeled by growth on phosphate-P^{32}. The activity of the cells was 116 c.p.m./10^9 cells. A, Cells exposed to 333 mcg./ml. of *p*-chloro-*m*-xylenol; B, saline-buffer control; C, ethanol (4.2% v/v)-buffer control.

TABLE VII.—EFFECT OF TEMPERATURE ON PHENOL, *p*-CHLORO-*m*-XYLENOL, AND *n*-BUTANOL-CAUSED RELEASE OF RADIOACTIVITY FROM LABELED *Escherichia coli* CELLS[a]

Treatment	Temperature, °C.	Radioactivity Released from Cells Labeled by Growth, % Glucose-C^{14}	Adenine-C^{14}	$Na_2S^{35}O_4$	$NaH_2P^{32}O_4$
Phenol, 12.5 mg./ml.	0	2.3	4.4	3.8	6.5
	22	4.8	14.6	11.0	18.4
	40	7.6	48.0	7.6	...
PCMX, 333 mcg./ml.	0	2.2	6.8	5.9	8.9
	22	3.5	7.0	7.1	17.9
	40	4.4	43.2	7.6	...
n-Butanol, 5% v/v	0	2.0	4.2	3.6	...
	22	6.6	8.2	14.2	...
	40	8.4	49.1	9.4	...
Ethanol (4.2% v/v)-buffer control	0	...	2.3	...	4.9
	22	...	3.1	...	5.6
	40	...	7.9	...	...
Saline-buffer control	0	0.7	2.1	0.7	2.5
	22	1.4	2.7	2.1	5.6
	40	1.9	5.8	1.6	...

[a] The labeled cells had approximately the following activities, c.p.m./10^9 cells: glucose-C^{14}, 269; adenine-C^{14}, 2656; sulfate-S^{35}, 1892; and phosphate-P^{35}, 40.

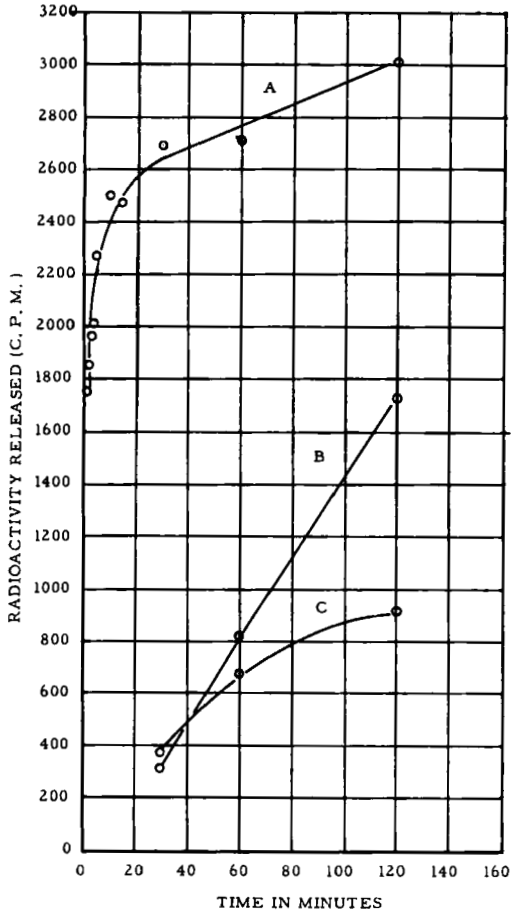

Fig. 2.—Release of radioactivity from *Escherichia coli* labeled by growth on sulfate-S^{35}. The cells had an activity of 1650 c.p.m./10^9 cells. A, Cells exposed to 333 mcg./ml. of *p*-chloro-*m*-xylenol; B, saline-buffer control; C, ethanol (4.2% v/v)-buffer control.

cause cytoplasmic degradation following membrane disruption. Bean and Walters (24) have shown that benzylchlorophenol-induced cell exudate from *E. coli* influenced survival in a bactericidal system and interpreted their data to indicate that the last survivors utilize the released cell constituents as nutrients. Also to be explored are the effects on metabolism of the germicidal derivatives of phenol, and even more challenging, the biochemical and biophysical basis for the relative nontoxicity to animals (25) of certain potent phenolic disinfectants such as *p*-chloro-*m*-xylenol and 2,4-dichloro-*m*-xylenol.

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Interaction of Pharmaceuticals with Schardinger Dextrins I

Interaction with Hydroxybenzoic Acids and *p*-Hydroxybenzoates

By JACK COHEN† and JOHN L. LACH

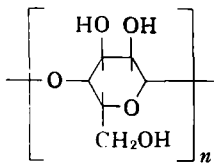
By means of the solubility method of analysis, the Schardinger dextrins were shown to interact in aqueous solution with benzoic acid derivatives. A definite interaction occurred with all compounds tested. Some stoichiometric data are presented and several formation constants calculated. It is postulated that both hydrogen bonding and inclusion formation are responsible for the main interaction observed.

SCHLENK (1) defines inclusion compounds as addition compounds in which one entity fits into and is surrounded by the crystal lattice of the other. These inclusion compounds may be called "no bond" complexes and are characterized by the lack of adhesive forces between the components of the complex. It is not a chemical interaction which causes an inclusion compound to form, although this may be a factor in the net complexation observed.

The steric configurations of the molecules are such that the enclosing, or "host," molecule can spatially enclose the included, or "guest," molecule, leaving unaffected the bonding systems of the components. Geometrical rather than chemical characteristics of the molecules are limiting factors in the interaction.

The chief forces involved in an inclusion compound are those holding host molecules to each other forming the space which encloses the guest molecule. Such is not the case with monomolecular inclusion compounds formed by one molecule of host enclosing one or more molecules of guest. This type of inclusion formation is typified by the Schardinger dextrins.

The Schardinger dextrins are a series of homologous oligosaccharides obtained from the breakdown of starch by the action of *Bacillus macerans* amylase. They are also known (2) as the cycloamyloses, cyclodextrins, and cycloglucans. They are homogeneous cyclic molecules composed of six or more alpha-D-glucopyranose units linked 1-4 as in amylose (3). Their structure is



where n is 6, 7, or 8, known as the alpha, beta, and gamma cyclodextrins, respectively.

The cyclic structure of the Schardinger dextrins confers upon the compound the ability to form monomolecular inclusion compounds where the guest is enclosed within the cyclodextrin void. These inclusion compounds may be grouped within the class of clathrate compounds where the host assumes a cagelike shape. Because of their cyclic structures, and the relatively large open space within each molecule (6 Å. for alpha, 8 Å. for beta, and 10–11 Å. for gamma), they have been reported to form complexes with crystal violet (4), carbon dioxide (5), 1-isopropyl azulene, cinnamaldehyde, hexachlorocyclohexane, pentamethylene tetrazole, and diethylaminoethyl-*p*-amino benzoate (6), ethylene (7), methyl orange (8), tetrachlorosalicylate, butter yellow, and adrenaline (9), and various gases (10).

Because of their different internal ring sizes, the cyclodextrins show different degrees of inclusion formation with different sized molecules. For example (11), alpha has been shown to form adducts with chlorine, bromine, iodine, hydrocarbons, and benzene derivatives with small substituents. Beta interacts with bromine, iodine, and almost any organic molecule. Gamma interacts with iodine and only large organic molecules. Alpha forms an inclusion compound with ethylene (7), while beta does not.

The clathrate forming power of the cyclodextrins has been utilized in various applications. They have been shown (12) to retard the cleavage of glycosides by inclusion. Cramer

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(13) has carried out basic catalyses with the cyclodextrins. They have been used as indicators (14) in paper chromatography. By means of the cyclodextrins (15), optically active compounds have been separated and solutions of labile compounds have been stabilized. They have been used for the resolution of racemates (16). The cyclodextrins form clathrates with many labile drugs such as unsaturated fatty acids and vitamin A (17). Since the drug included is, in essence, shielded by the host from its surroundings, stability of the drug can be greatly increased.

In view of the properties of these dextrins, this study was undertaken as a preliminary investigation into the possible utilization of the Schardinger dextrins in pharmaceutical systems.

EXPERIMENTAL

Reagents.—Benzoic acid, recrystallized, m.p. 119–121°; *o*-hydroxybenzoic acid, recrystallized, m.p. 155–157°; *m*-hydroxybenzoic acid, recrystallized, m.p. 198–200°; *p*-hydroxybenzoic acid, recrystallized, m.p. 212–214°; methylparaben, m.p. 124–126°; ethylparaben, recrystallized, m.p. 112–115°; propylparaben, recrystallized, m.p. 95–97°; butyl paraben, recrystallized, m.p. 67–69°; alpha cyclodextrin; beta cyclodextrin; *Bacillus macerans*, strain 7069, American Type Culture Collection; agar; dextrose; nutrient broth; Quaker oats; calcium carbonate, reagent grade; potato starch; trichloroethylene; bromobenzene; and *n*-propanol were used as reagents.

Apparatus.—A constant temperature water bath, set at 30°, ±1/2°, with rotating spindle; 20-ml. capacity vials with gum rubber stoppers and aluminum caps; Beckman DU spectrophotometer; 1-cm. and 5-cm. silica cells comprised the necessary apparatus.

PROCEDURE

Preparation of the Schardinger Dextrins.—The Schardinger dextrins are prepared by the action of *Bacillus macerans* amylase on one or another of the common starches.

Our method of preparation was based on the experiences of others, notably French and his group (18) and Tilden and Hudson (19). The complete process is quite involved and time consuming and need not be gone into in detail, although the basic procedure will be summarized.

Bacillus macerans was grown on a rolled oats medium, buffered with calcium carbonate, at 40° for 2 to 3 weeks to permit production of the amylase enzyme. This enzyme-containing solution was then decanted into 4% starch (potato) paste and stored, with intermittent agitation, at 40° for 24 hours. At the end of this period, marked by a noticeable decrease in the viscosity of the starch paste, the production of the Schardinger dextrins was completed.

The Schardinger dextrins were precipitated out of solution as trichloroethylene complexes. The alpha, beta, and gamma cyclodextrins were then separated by taking advantage of their differing solubilities in water and in the presence of bromobenzene. The individual cyclodextrins were then purified by repeated crystallizations from water and 60% *n*-propanol.

The greatest yields obtained were of beta, with lesser but satisfactory yields of alpha. Gamma was produced in such small quantities that no experimentation with this cyclodextrin was possible.

The cyclodextrins were checked for purity by the microscopic method of French (18) and by carbon-hydrogen analysis (Table I). These were then dried in an oven at 70° and kept in a desiccator until used.

TABLE I.—CARBON-HYDROGEN ANALYSIS OF THE CYCLODEXTRINS

Cyclodextrin	% C Calcd.	% C Det.	% H Calcd.	% H Det.
Alpha	44.44	43.79	6.22	6.16
Beta	44.44	43.84	6.22	6.46

Solutions of the cyclodextrins were freshly prepared, with the aid of heat, before each run because of the tendency toward mold growth in these solutions.

Solubility Studies.—The procedure used in this study was the solubility method of Higuchi and Lach (20).

Quantities in excess of the normal solubilities of the drugs studied were weighed into 20-ml. vials. Quantities of 3% alpha or 1% beta cyclodextrin were accurately measured in, and water added to make a volume of 5 ml. of solution in each vial. A more concentrated solution of beta was not used because of its limited solubility in water. The vials were sealed with gum-rubber stoppers and aluminum caps and rotated on a mechanical shaker in a constant temperature water bath at 30° for 24–48 hours until the system reached equilibrium.

At the end of the equilibration time, 1- or 2-ml. aliquots of the supernatant solutions were removed, diluted with water, and analyzed for total drug concentration by ultraviolet spectrophotometry. Since several of the vials showed solid material present, a cotton plug was used as a filter on the pipet tips.

Absorptivities had previously been determined for each of the drugs studied at the following wavelengths: benzoic acid, 272.5 mμ; *o*-hydroxybenzoic acid, 298 mμ; *m*-hydroxybenzoic acid, 290 mμ; *p*-hydroxybenzoic acid, 251 mμ; methyl *p*-hydroxybenzoate, 256 mμ; ethyl *p*-hydroxybenzoate, 256 mμ; propyl *p*-hydroxybenzoate, 255 mμ; and butyl *p*-hydroxybenzoate, 256 mμ. It was determined that the cyclodextrins did not interfere with the spectrophotometric measurements at the concentrations employed.

RESULTS AND DISCUSSION

In order that some insight be gained into the mechanism of interaction of organic compounds with the Schardinger dextrins, two homologous series of acid derivatives, the hydroxybenzoic acids and the *p*-hydroxybenzoates, were tested for complexing activity with the cyclodextrins.

A definite interaction occurred with each of these eight compounds with both alpha and beta cyclodextrins. The straight-line plots obtained show that the increase in solubility of the drug is a function of the concentration of the cyclodextrin. All of the isotherms show a positive slope and several show a plateau. Plots showing this plateau are indicative of a relatively insoluble complex being formed. Phase diagrams of this type, with calculation of stoichiometries and formation constants, have been explained by Higuchi and Lach (20).

The Hydroxybenzoic Acids.—Figures 1 and 2 represent interactions of the hydroxybenzoic acids with the cyclodextrins. Table II represents the slopes of these plots. Several conclusions can be drawn from these data.

TABLE II.—SLOPES OF ISOTHERMS OF INTERACTIONS OF THE HYDROXYBENZOIC ACIDS WITH THE SCHARDINGER DEXTRINS

	Alpha	Beta
o-Hydroxybenzoic acid	0.305	0.885
p-Hydroxybenzoic acid	0.813	1.044
Benzoic acid	1.004	1.135
m-Hydroxybenzoic acid	1.085	1.192

Data for these interactions indicate no clear cut mechanism of complexation. If these interactions were pure, that is classic, inclusion formation, the smallest molecule, unsubstituted benzoic acid, would show the greatest degree of inclusion and therefore the greatest slope. Since it does not, other forces are probably involved. This is reasonable in view of the multiplicity of hydroxyl groups on the cyclodextrin molecule and the likelihood of hydrogen bonding between the cyclodextrin and the hydroxybenzoic acids in the aqueous system employed. This is further substantiated by the lowest complexing activity, as evidenced by the lowest slope, being a property of the *ortho*-hydroxy acid in the case of both alpha and beta interaction. *ortho*-Hydroxybenzoic acid possesses intramolecular hydrogen bonds, thus decreasing the number of hydroxyl and carboxyl groups available for hydrogen bonding with the cyclodextrin. Hydrogen bonding therefore would seem to be a factor of importance in the cyclodextrin-hydroxybenzoic acid interaction, especially since we note that the *meta*- and *para*-hydroxy acids interact to approximately the same degree as benzoic acid itself. Since these substituted acids are larger than the parent acid, we would expect them to show less tendency towards inclusion formation. However, they possess an extra hydroxyl group, causing greater intermolecular hydrogen bonding with the cyclodextrins, and a greater degree of interaction than expected is observed.

It would seem, therefore, that the interactions observed are not true inclusion formation. Inclusion formation as such involves molecules associated by spatial relationships only, while in the interactions observed, hydrogen bonding seems to play a significant role. Thus the interaction data must be considered from a semiquantitative standpoint since the interaction is probably a result of more than one mechanism; attempts to interpret the data assuming inclusion formation only would not be wholly successful.

Results of Schlenk and Sand (21) support this conclusion. Their work on the association of the Schardinger dextrins with organic acids showed that the mole ratio of the cyclodextrins to certain benzoic acid derivatives was a function of the physical state of the complex. The stoichiometry of

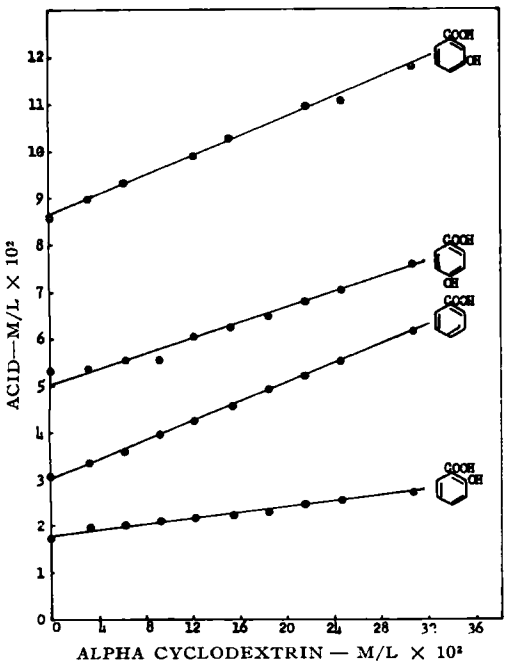

Fig. 1.—Interaction of the hydroxybenzoic acids with alpha cyclodextrin at 30°.

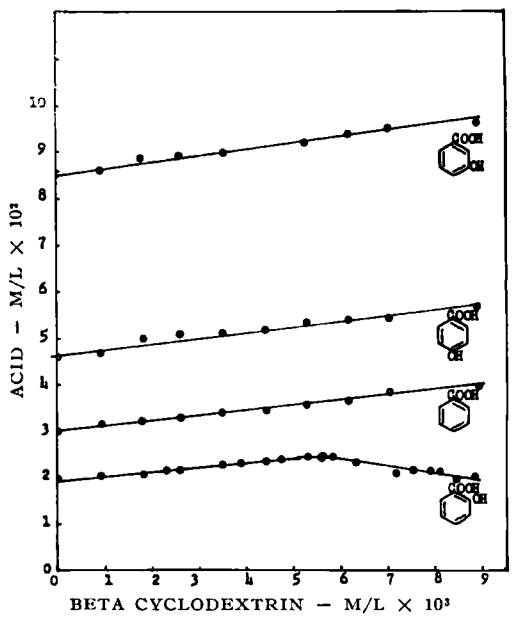

Fig. 2.—Interaction of the hydroxybenzoic acids with beta cyclodextrin at 30°.

the crystallized solid complex was not the same as that of the associated compound in solution. This indicates a complex mechanism of interaction in the aqueous system studied. It should be emphasized here that this study was conducted in aqueous solution where conditions for hydrogen bonding are favorable. In nonpolar systems, hydrogen bonding would not be as significant a factor in the net interaction.

Certain plots obtained through solubility data provide information concerning both stoichiometry and formation constant of the complex formed.

Where no plateau region is obtained, stoichiometry cannot be determined because the concentration of free drug present in solution is an invariant. However, linear dependence of the complexation upon cyclodextrin concentration is indicative of a first order dependency of the interaction upon cyclodextrin concentration. In cases such as this, stoichiometry with respect to the drug molecule is difficult to calculate since the data were obtained at constant drug activity because of the drug excess present.

Of the hydroxybenzoic acids, only the salicylic acid-beta cyclodextrin plots shows a plateau region which is indicative of formation of a relatively insoluble complex. From the plateau region of a phase diagram of this type, the stoichiometry of the complex can be determined and its formation constant calculated (20). Analysis of this region indicates three salicylic acid to one beta cyclodextrin complex, with a formation constant on the order of 5×10^4. This value is accepted with reservation because of the probable complexity of the interaction. The magnitude of this formation constant does indicate, however, the existence of a complex of great stability. In other studies of interactions of the hydroxybenzoic acids (22–25), calculated formation constants were all on the order of 1–100, calculated on the basis of a 1:1 interaction. Since the cyclodextrin interaction constant is much higher, an interaction of greater stability than the previously reported intermolecular attractive forces is indicated. This is reasonable in view of the complexity of these interactions and the probability of more than one interaction mechanism being involved.

The *p*-Hydroxybenzoates.—In the study of the interactions of the cyclodextrins with esters of *p*-hydroxybenzoic acid, strong interactions are also observed (Figs. 3 and 4).

Alpha forms soluble complexes with the parabens while the beta isomer forms complexes of limited solubility. The complexes formed with alpha are all soluble due to the greater solubility of alpha over beta. For the beta interactions, the data indicate that the less the solubility of the paraben, the less the solubility of the complex. Thus, methyl forms a soluble complex while butyl forms a relatively insoluble complex. The ethylparaben and propylparaben complexes show intermediate stages of solubility.

For both alpha and beta interactions with the parabens, a decrease in slope with increasing molecular weight is observed, except for the propyl and butyl esters (Table III). This latter phenomenon

TABLE III.—SLOPES OF ISOTHERMS OF INTERACTIONS OF THE *p*-HYDROXYBENZOATES WITH THE SCHARDINGER DEXTRINS

	Alpha	Beta
Methylparaben	0.911	1.044
Ethylparaben	0.510	0.896
Propylparaben	0.295	0.687
Butylparaben	0.357	0.747

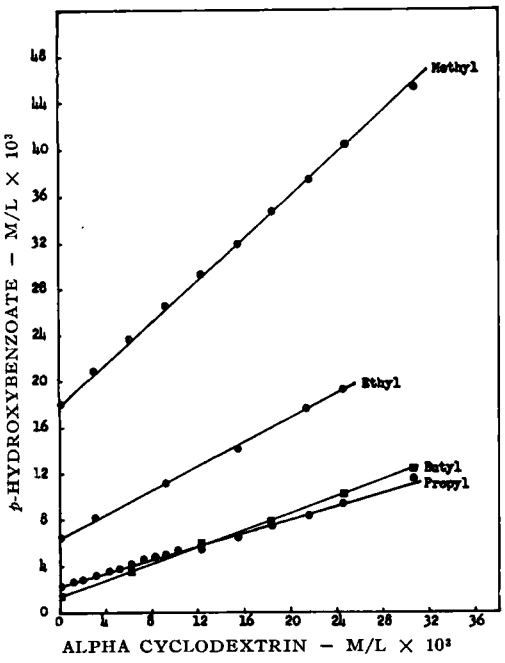

Fig. 3.—Interaction of the *p*-hydroxybenzoates with alpha cyclodextrin at 30°.

requires further elucidation of the mechanism of interaction before any explanations can be attempted. The overall tendency towards generally decreasing slope can be attributed to spatial filling; as the ester chain becomes longer and the molecule therefore larger, less inclusion of the ester takes place.

Calculation of stoichiometry along the propyl-beta and butyl-beta plateaus shows a molecular ratio in each case of 1:1. On this basis, stoichiometries of the methyl-beta and ethyl-beta complexes are tacitly assumed to be 1:1, and formation constants calculated (Table IV).

TABLE IV.—FORMATION CONSTANTS OF THE PARABEN-BETA COMPLEXES

Compound	K_f
Methylparaben	"infinite"
Ethylparaben	1.38×10^3
Propylparaben	9.23×10^2
Butylparaben	2.04×10^3

These formation constants are quite high compared to those previously reported in pharmaceutical interaction studies. Stability constants reported for paraben complexes at 30° are on the order of 50 for complexes with caffeine (21), and are generally below 50 for complexes of drugs with other complex-

ing agents when studied by the solubility method (26). The work of Poole and Higuchi (27) with sarcosine anhydride shows very low formation constants, with some values approaching the magnitude of our results but usually falling below 100.

Formation constants of the magnitude reported here indicate formation of a complex of increased stability. Thus an interaction involving more than hydrogen bonding or dipole-dipole attraction is likely involved and a combination of these plus inclusion formation is a distinct probability.

Unpublished results of Pauli (28) indicate that alpha cyclodextrin has a greater stabilizing effect on the stability of benzocaine to alkaline hydrolysis than any other complexing agent reported. This too is indicative of a stable complex and lends additional support to the above conclusions.

CONCLUSION

Molecular complexes exist where there are forces of an electrostatic nature drawing molecules together. Inclusion formation involves molecules held together by virtue of their spatial configurations; these compounds have been defined as being chemically inert toward each other since true inclusion formation involves no attractive forces between guest and host.

This criterion for inclusion formation may be valid for crystalline and solid complexes, but when the complex exists in aqueous solution, as in this study, attractive forces such as hydrogen bonds come into play. Evidence for this is provided by the hydroxybenzoic acid interactions where hydrogen bonding appears to play a significant role in the net interaction observed. Therefore, in these interactions of the cyclodextrins with organic molecules, we assume that hydrogen or other attractive bonds act to bring the molecules together in solution and play a part in holding them together.

The net interaction observed in this study is believed to be a combination of attractive forces and inclusion formation. No distinctions as to the relative contributions of the two forces can be obtained from the data presented. Since X-ray examination of solid compositions is the only method known by which unequivocal identification of inclusion compounds can be made (29), this question is deferred for further study.

High formation constants observed in this study indicate a more stable complex than is usually observed in pharmaceutical studies of this type. Since, undoubtedly, some attractive forces are to be considered in this study, we feel that the interaction of the Schardinger dextrins with organic molecules is probably a combination of hydrogen bonding, inclusion formation, or other forces acting as factors in the net observed interaction.

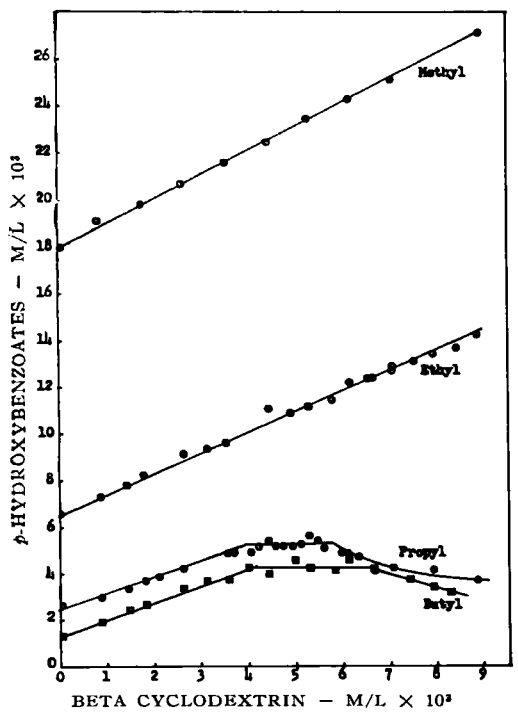

Fig. 4.—Interaction of the *p*-hydroxybenzoates with beta cyclodextrin at 30°.

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Interaction of Pharmaceuticals with Schardinger Dextrins II

Interaction with Selected Compounds

By JOHN L. LACH and JACK COHEN†

Data for the interaction of the Schardinger dextrins with 19 pharmaceuticals in aqueous solution are presented. All the compounds interact with both alpha and beta cyclodextrin. Data are presented to show that the less soluble the drug is in water, the greater is its per cent increase in solubility as a function of cyclodextrin concentration. Comparison of the isothermal slopes of the interactions shows that the largest molecules show the smallest slopes and therefore the smallest degree of interaction with the cyclodextrins. Molecular structure is a very important factor in the interaction which is believed to be a combination of inclusion formation and other intermolecular attractive forces.

THE PHENOMENON of complexation has become a very active area of pharmaceutical research. Only during this past decade has its importance in pharmaceutical formulations been fully realized. Many varied types of molecules and macromolecules have been found to interact with drug molecules of widely differing structures. Beginning with the work of Higuchi and Zuck (1), pharmaceutical researchers have devoted a good deal of time to elucidation of the nature of these phenomena.

Solubility changes have been observed as a function of these interactions and substantial increases in drug stability have been reported (2–6). Of particular importance in pharmacy and medicine is the relationship of complexation to the absorption and distribution of a drug in the body and the way that complexation influences the onset and duration of drug action (7).

In Part I of this series (8) it was observed that the Schardinger dextrins form complexes with the hydroxybenzoic acids and the *p*-hydroxybenzoates. This work was an attempt to extend the study to a variety of pharmaceutical compounds to obtain additional information concerning this interaction.

EXPERIMENTAL

The apparatus and reagents used in this study were those reported in Part I of this series, except that the following drugs were tested for complexing activity; benzocaine, m.p. 87–88°; aspirin, m.p. 134–135°; tetracycline, m.p. 170–173°; *p*-aminobenzoic acid, m.p. 185–186°; sulfadiazine, m.p. 255–256°; morphine, m.p. 250°; vanillin, m.p. 78–81°; N-acetyl-*p*-aminophenol, m.p. 169–170°; *p*-aminosalicylic acid, m.p. 150–151°; ephedrine, m.p. 34°; and sorbic acid, m.p. 134–138°.

The Schardinger dextrins were prepared by accepted methods (9, 10) and the interactions studied by the solubility method of Higuchi and Lach (11). The drugs were analyzed spectrophotometrically at the following wavelengths: benzocaine, 285 mμ; aspirin, 275 mμ; tetracycline, 357 mμ; *p*-aminobenzoic acid, 278 mμ; sulfadiazine, 266 mμ; morphine, 285 mμ; vanillin, 279 mμ; N-acetyl-*p*-aminophenol, 244 mμ; *p*-aminosalicylic acid, 267 mμ; ephedrine, 257 mμ; and sorbic acid, 259 mμ.

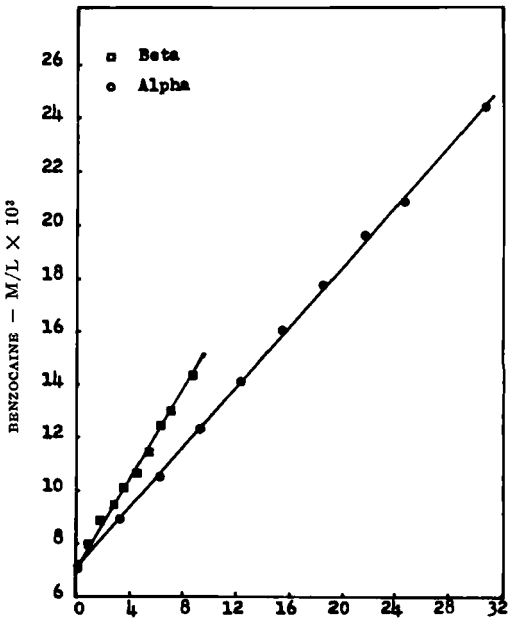

Fig. 1.—Interaction of benzocaine with the cyclodextrins at 30°.

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Aspirin was run in 10^{-3} *M* sulfuric acid to decrease its rate of hydrolysis.

RESULTS AND DISCUSSION

Figures 1–11 represent solubility isotherms of the interactions of 11 drugs with the alpha and beta cyclodextrins. The Schardinger dextrins interact with all compounds tested. Linear plots are obtained with most drugs and both cyclodextrins, demonstrating first order dependence of the inter-

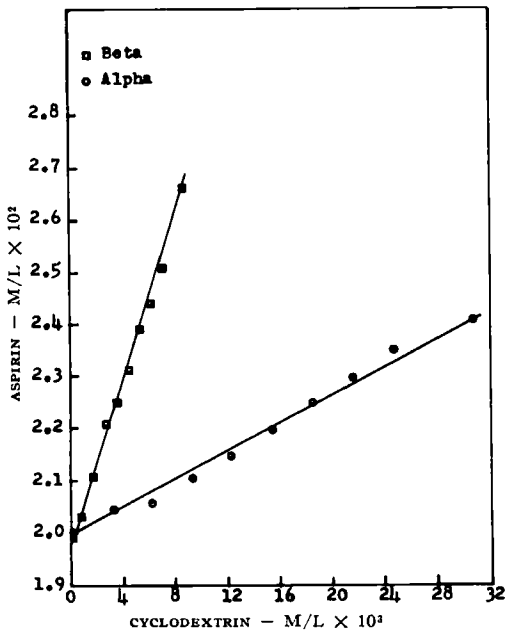

Fig. 2.—Interaction of aspirin with the cyclodextrins at 30°.

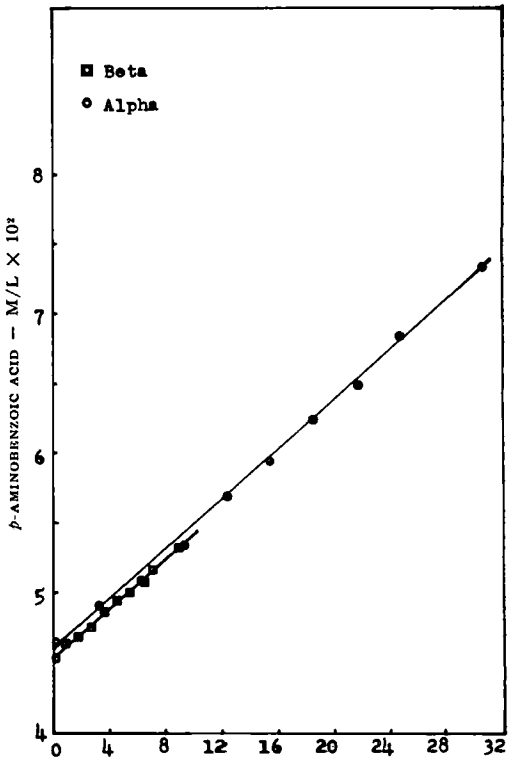

Fig. 4.—Interaction of *p*-aminobenzoic acid with the cyclodextrins at 30°.

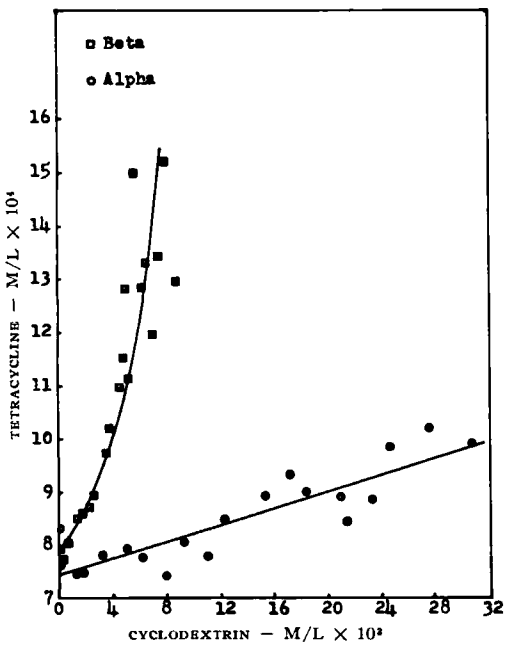

Fig. 3.—Interaction of tetracycline with the cyclodextrins at 30°.

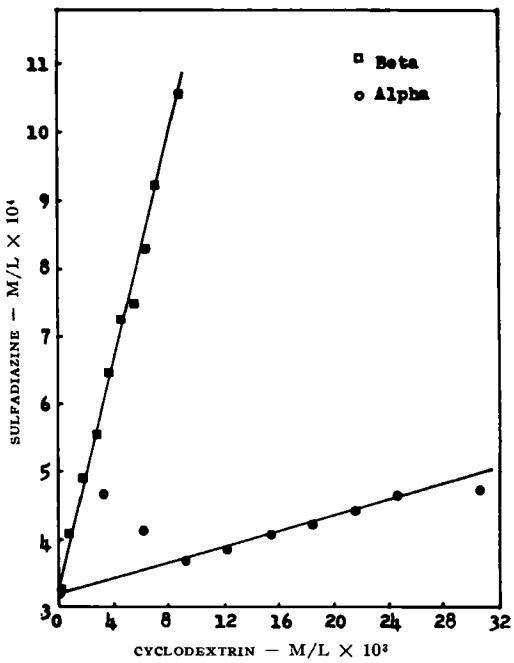

Fig. 5.—Interaction of sulfadiazine with the cyclodextrins at 30°.

action upon cyclodextrin concentration. The morphine-beta and tetracycline-beta plots show a curvature, probably due to the nature of the complex (which prevented complete filtration) or to unperceived complexities in the interaction. The tables and figures presented also include some data from Part I of this series (8), for comparative purposes.

All the compounds studied, except morphine, tetracycline, sulfadiazine, and sorbic acid, are substituted benzene rings. Benzene rings will fit into the voids of both alpha and beta cyclodextrins since the dextrin voids are 6 and 8 Å. in diameter, respectively, while the phenyl structure occupies a space measuring approximately 7 × 7 × 3.4 Å. (12, 13).

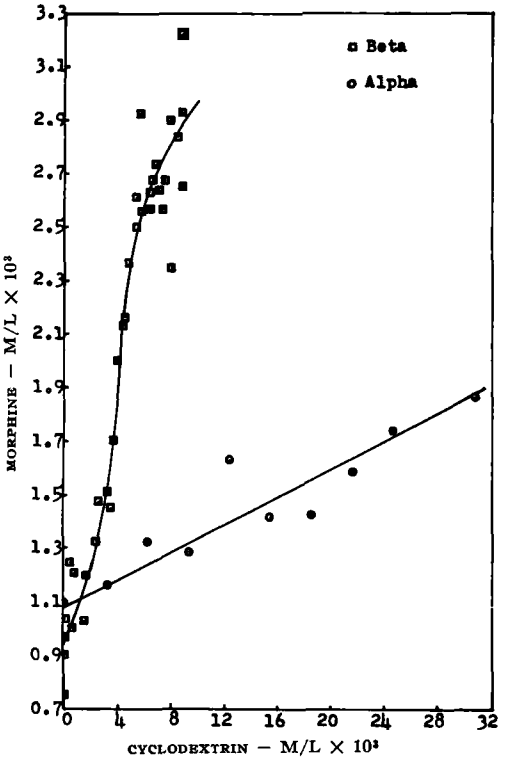

Fig. 6.—Interaction of morphine with the cyclodextrins at 30°.

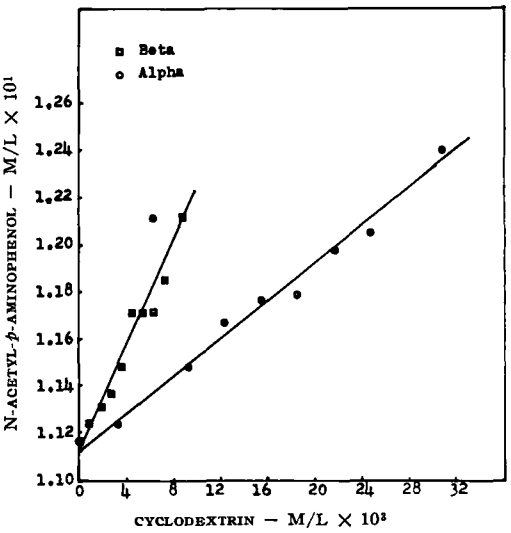

Fig. 8.—Interaction of *N*-acetyl-*p*-aminophenol with the cyclodextrins at 30°.

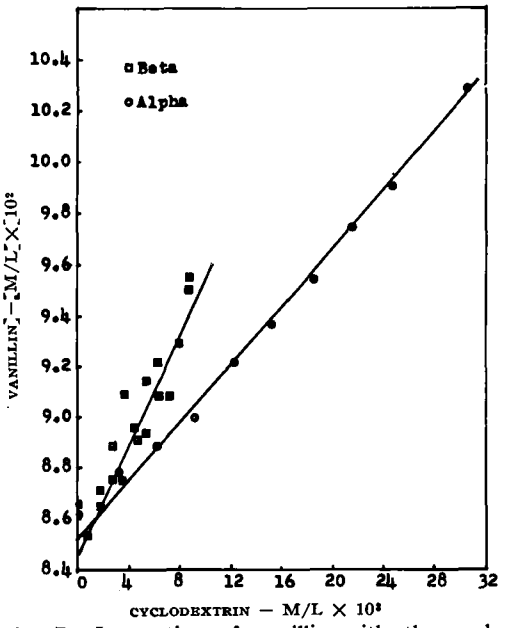

Fig. 7.—Interaction of vanillin with the cyclodextrins at 30°.

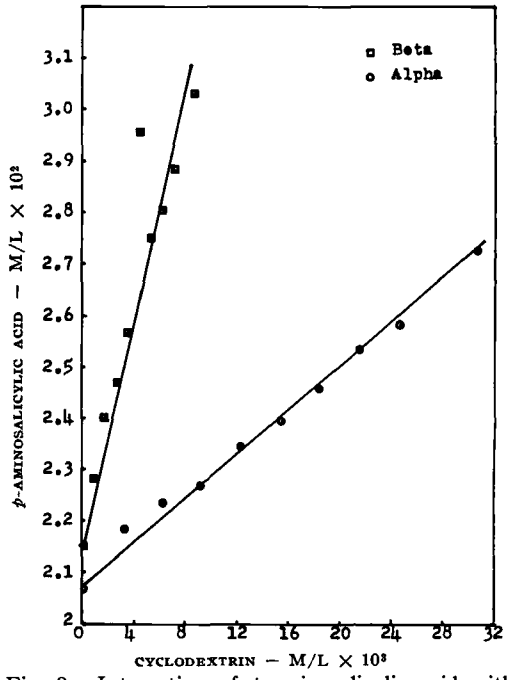

Fig. 9.—Interaction of *p*-aminosalicylic acid with the cyclodextrins at 30°.

Allowing for slight molecular structural changes caused by inclusion formation and other distorting forces, it is apparent that these molecules would be prone to inclusion by the alpha and beta cyclodextrins.

Morphine, tetracycline, and sulfadiazine, being polynuclear ring systems, are quite large compared to the phenyl group and fit into the voids with difficulty. If we assume that the others are complexed, in part, by inclusion formation, we can see that these three, being too large for inclusion, cannot interact in this way to any significant degree.

It is possible, however, that side chains and substituent groups may fit into the dextrin void and that the complexation is actually a matter of these groups being included in the void, thereby forming the inclusion complex. Since questions of this sort concern exact structural conformation of the complex, which can be determined only by X-ray diffraction, elucidation of this mechanism is postponed until further study.

Several methods have been used to compare relative complexing tendencies using data obtained from solubility studies. Comparison of the per cent increase in solubility has been used (14) as a comparison of relative complexing tendencies with the assumption being made that a higher per cent increase in solubility involves a greater degree of interaction. In this study it was felt that a truer picture of relative complexing tendencies could be obtained by comparing the slopes of the interaction isotherms.

In phase diagrams showing a plateau region, stoichiometries and formation constants can be calculated and overall complexing tendencies compared by interpretation of these constants. Since, in this study, no plateau regions were observed, slopes of the interaction isotherms are used as indications of relative complexing tendencies (Table I).

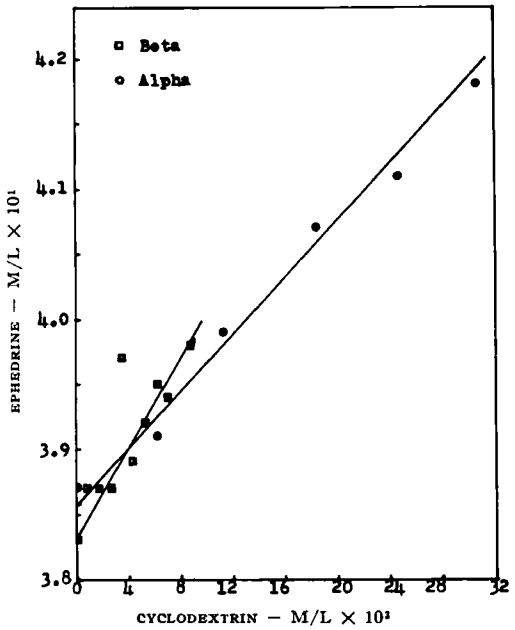

Fig. 10.—Interaction of ephedrine with the cyclodextrins at 30°.

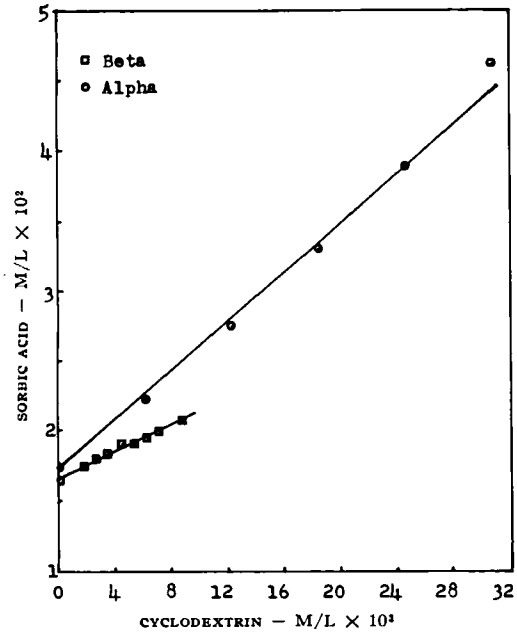

Fig. 11.—Interaction of sorbic acid with the cyclodextrins at 30°.

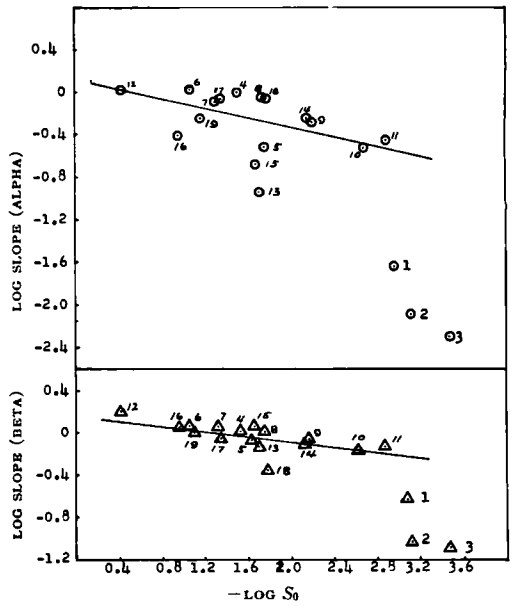

Fig. 12.—Relationship of slope of interaction isotherms of drugs with the cyclodextrins to initial solubility at 30°. Key: 1, morphine; 2, tetracycline; 3, sulfadiazine; 4, benzoic acid; 5, salicylic acid; 6, *m*-hydroxybenzoic acid; 7, *p*-hydroxybenzoic acid; 8, methylparaben; 9, ethylparaben; 10, propylparaben; 11, butylparaben; 12, ephedrine; 13, acetylsalicylic acid; 14, benzocaine; 15, *p*-aminosalicylic acid; 16, N-acetyl-*p*-aminophenol; 17, *p*-aminobenzoic acid; 18, sorbic acid; and 19, vanillin.

TABLE I.—SLOPES OF INTERACTION ISOTHERMS OF 19 COMPOUNDS WITH ALPHA AND BETA CYCLODEXTRIN

Alpha		Beta	
Drug	Slope	Slope	Drug
Sulfadiazine	0.00519	0.0845	Sulfadiazine
Tetracycline	0.0081	0.0937	Tetracycline
Morphine	0.025	0.237	Morphine
Aspirin	0.133	0.454	Sorbic acid
p-Aminosalicylic acid	0.211	0.687	Propylparaben
Propylparaben	0.295	0.747	Butylparaben
Salicylic acid	0.305	0.772	Aspirin
Butylparaben	0.357	0.795	Benzocaine
N-Acetyl-*p*-aminophenol	0.395	0.891	*p*-Aminobenzoic acid
Ethylparaben	0.510	0.885	Salicylic acid
Benzocaine	0.567	0.896	Ethylparaben
Vanillin	0.567	1.010	Vanillin
p-Hydroxybenzoic acid	0.813	1.044	Benzoic acid
p-Aminobenzoic acid	0.891	1.044	Methylparaben
Methylparaben	0.911	1.100	N-Acetyl-*p*-aminophenol
Sorbic acid	0.911	1.111	*p*-Aminosalicylic acid
Benzoic acid	1.004	1.135	*p*-Hydroxybenzoic acid
m-Hydroxybenzoic acid	1.085	1.192	*m*-Hydroxybenzoic acid
Ephedrine	1.101	1.588	Ephedrine

However, it must be realized that this comparison of slopes is an approximation. If complexes show the same stoichiometries, comparison of their slopes directly relates stability constants of the interactions. Since these stoichiometries are unknown, due to the nature of the phase diagram, comparison of slopes of interactions of different stoichiometries can lead to errors in interpretation. Thus, the correlation is accepted as an approximation and is used because of the lack of exact stoichiometric data.

Inspection of Table I shows that the largest molecules show the smallest slopes. Their solubilization by the cyclodextrins, relative to their initial solubilities, is great but the extent of complex formation with these compounds is small.

In general, the smallest and most soluble drugs show the highest slopes. Since compounds of lower molecular weight are usually more water soluble, one is a corollary of the other. A plot of log slope *vs.* log S_o (solubility in absence of dextrin), Fig. 12, shows fair regularity of this trend. In view of the part played by inclusion formation in this interaction, we feel that the smaller molecules show a greater inclusion tendency because of greater ease of fitting into the dextrin voids. Morphine, tetracycline, and sulfadiazine show deviation from this linearity, presumably caused by the decrease in inclusion formation tendency because of their large sizes. These large molecules show interaction with the cyclodextrins even though they are too large to be guests in the dextrin ring. Hydrogen bonding, due to the multiplicity of hydroxyl and carboxyl groups on the cyclodextrin ring, occurring in aqueous solution, is probably the chief interaction mechanism here and is responsible for solubilization of the larger molecules.

Most of the compounds tested show a greater slope for beta interaction than for alpha interaction with a few compounds showing the same slope for both. This greater interaction with beta is reasonable in that the beta void is larger than the alpha void and can include molecules to a greater degree. The compounds studied, being phenyl derivatives, fit more easily into the beta void than the alpha void since the alpha void is of approximately the same size as the diameter of the benzene ring.

Sorbic acid was the only straight chain compound studied. This is the only compound studied where the complexing activity with alpha is greater than that with beta. Here the chain molecule fits into the alpha void with ease, unlike compounds containing one benzene ring where some strain is necessarily involved by inclusion formation with alpha. It is also possible that the chain molecule is too small in diameter to form a stable complex with beta cyclodextrin since a minimum size of guest is necessary for stable inclusion formation. If a guest is too small, forces holding it within the void are too weak to form a stable complex. This reverse of previous observations by sorbic acid is logical evidence of the existence of strong inclusion formation by the compounds studied.

Most studies of inclusion formation have been carried out in nonaqueous media. It is felt that a study of this type conducted in nonaqueous media would yield additional information as to the mechanism of interaction and estimates of the relative contributions of molecular complexing and inclusion formation could be made. A study of this type is currently under way in our laboratories.

CONCLUSION

The data in this study indicate that the Schardinger dextrins form complexes with a variety of pharmaceutical compounds. It was observed that those drugs which are least soluble in water showed the greatest per cent increase in solubility as a function of quantity of cyclodextrin present. In general, the smallest drug molecules showed the greatest complexing activity with the cyclodextrins. Interaction with sorbic acid demonstrates the importance

of the structure of the guest molecule to the degree of interaction where inclusion formation is a major contributing mechanism.

Molecules which are too large to be included within the cyclodextrin voids interact with the cyclodextrins indicating an interaction mechanism other than inclusion formation. The literature dealing with inclusion formation by various host molecules usually considers only inclusion formation as a mechanism of interaction for these compounds. However, in this aqueous system, we believe that other intermolecular attractive forces, particularly hydrogen bonds, seem to play a part in the net observed interaction.

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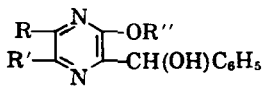
By VINCENT S. VENTURELLA†, J. A. BIANCULLI, and R. W. SAGER‡

Several derivatives of 2-hydroxypyrazine were prepared containing a phenylcarbinol moiety in the 3-position. A method was developed to prepare these compounds in order to test the efficacy of the basic cyclization reaction between a bifunctional α-amino amide and several 1,2-dicarbonyls. The synthetic method used necessitated some study of the chemistry of threo-β-phenylserine and its amide. After many unsuccessful experiments, it was found that the amide of this acid is best prepared through the use of the N-carbobenzoxy methyl ester.

THE PURPOSE of this investigation was to prepare several compounds of general structure, I, which might be useful as psychopharmacological agents.



	R	*R'*	*R''*
I	H	H	H
	H	H	CH_3
	H	CH_3	H
	H	CH_3	CH_3
	CH_3	CH_3	H
	CH_3	CH_3	CH_3
	C_6H_5	C_6H_5	H
	C_6H_5	C_6H_5	CH_3

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Abstracted from a thesis submitted to the Graduate Faculty of the University of Pittsburgh, School of Pharmacy, by V. S. Venturella in partial fulfillment of the requirements for the Ph.D. degree.
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‡ Present address: Oregon State University, College of Pharmacy, Corvallis.

In the event that two nitrogen functions (as present in serotonin) and added hydroxyl functions (1) are necessary for antimetabolite activity, these compounds should exhibit some activity toward blocking or reversing the physiological activity of excess serotonin (2, 3).

DISCUSSION

Several methods have previously been used for the preparation of substituted hydroxypyrazines (4, 5), but none of these methods was applicable to the introduction of the phenylcarbinol group into the 3-position of the pyrazine ring.

The most promising methods available were those developed by Sharp and Spring (6) and by Jones (7). The Jones method was chosen for the synthesis of the cyclized product because the former method would require the use of the nitrile of β-phenylserine as an intermediate. The preparation of this compound, although possible, was expected to result in a great deal of difficulty because of the presence of the β-hydroxy group. The Jones method for the final step in the synthesis is represented in Eq. 1

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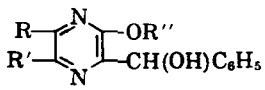

	R	*R'*	*R''*
I	H	H	H
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	H	CH_3	H
	H	CH_3	CH_3
	CH_3	CH_3	H
	CH_3	CH_3	CH_3
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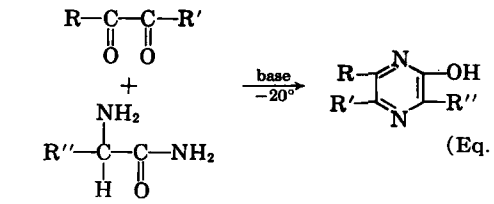
The most promising methods available were those developed by Sharp and Spring (6) and by Jones (7). The Jones method was chosen for the synthesis of the cyclized product because the former method would require the use of the nitrile of β-phenylserine as an intermediate. The preparation of this compound, although possible, was expected to result in a great deal of difficulty because of the presence of the β-hydroxy group. The Jones method for the final step in the synthesis is represented in Eq. 1



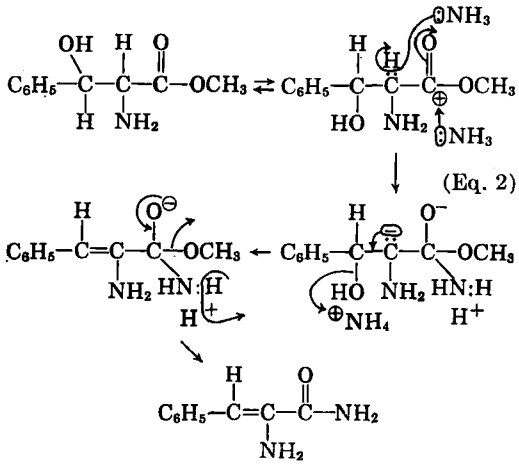
(Eq. 1)

The use of this method for the final step therefore required the use of one of the isomers of phenylserine due to R″ = $C_6H_5CH(OH)$—. The threo isomer was used throughout the experiments due to the possible steric interference that might result from the use of the erythro compound in the Jones sequence.

The isomerically pure acid was prepared by a modification of the procedure of Shaw and Fox (8) and its isomeric purity ascertained chromatographically (9) by comparison with authentic samples of the acids.[1]

The lower esters of the acid resisted ammonolysis using standard procedures, and treatment of the ethyl ester hydrochloride with liquid ammonia yielded only the erythro amide which gave an R_f value of 0.82 when chromatographed in a manner similar to the acid. The stereochemistry of this compound was demonstrated by infrared analysis which showed an absorption in the 11.9 to 12.1 μ range, which is indicative of the erythro isomers of this general series of compounds (10, 11). The absence of bands at 6.2 and 11 μ precludes the possibility that this compound was recovered β-phenylserine, which was suggested by the proximity of melting points.

When the ammonolysis of the methyl ester was attempted under pressure with continuous agitation over extended periods of time, a compound was isolated which was shown to be α-aminocinnamamide. The formation of this compound may be explained by the sequence of reactions in Eq. 2



(Eq. 2)

The infrared spectrum of this compound showed strong bands in the $—NH_2$ and $—CONH_2$ regions, presence of monophenyl substitution, absence of —CH(OH)—, and a conjugated carbonyl system, as evidenced by the shift in the carbonyl absorption followed by a doublet (12, 13).

Since previous attempts to prepare the threo amide were unsuccessful or resulted in isomerization, the electromeric carbobenzoxy group (14) was used to reverse the inductive effect of the amino group. This reversal permitted an easier discharge of the $—OCH_3$ group due to activation of the ester group followed by an unhindered attack of $:NH_3$, according to the inductomeric mechanism (15). The carbobenzoxy group prevents neighboring group neutralization of the carbonyl carbon as well as providing activation of the ester group.

The relationship of stability of isomers to favored formation of amide can be related to configuration versus reaction specificity. The erythro form is less stable, but its formation is favored due to the possibility of hydrogen bonding between the α-amino and the β-hydroxy groups. The relatively more stable threo isomer is made to form by the presence of the bulky carbobenzoxy group which sterically removes the possibility of inversion and electronically removes the possibility for hydrogen bonding.

Condensation Reactions.—Threo-β-phenylserine amide was reacted with the 1,2-dicarbonyls studied using a modification of the Jones procedure for the reaction of tyrosine amide (7). An excess of the dicarbonyl was used in the reaction in all cases except pyruvic aldehyde condensation. With this compound it was found that using an excess of the amide resulted in greater yield, purer product, and easier isolation of product.

Derivative Formation.—It has been shown (16) that compounds of this type exist in tautomeric equilibrium with the corresponding pyrazone structures. This phenomenon was further demonstrated during this study by the strong infrared absorption bands in the carbonyl region of the prepared compounds. The presence of this group along with the coexistence of the functional nuclear hydroxyl group and the side chain hydroxyl group suggested the possibility of hydroxylic and ketonic derivatives.

However, it was found that steric crowding prevented the formation of hydroxylic derivatives, phenylhydrazide and semicarbazide. In contrast to this, the compounds reacted in low yield in aqueous dimethylsulfate to form the corresponding methyl ethers of the nuclear hydroxyl groups.

EXPERIMENTAL[2]

Threo-β-phenylserine Monohydrate.—The procedure of Shaw and Fox (8) was followed for the preparation of this compound, with minor variations. Twenty-five Gm. (0.33 mole) of glycine in 100 ml. of 6 *N* sodium hydroxide was cooled to 10° and stirred vigorously while 70 Gm. (0.66 mole) of benzaldehyde was added slowly. When the resultant slurry thickened, 20 ml. of water was added and the thick edges were broken up. At this point the benzaldehyde addition was stopped and the slurry was stirred very vigorously until it was homogeneous. The benzaldehyde addition was then completed. The mixture was stirred for a half hour at 10°, and finally at room temperature until pasty. After standing at room temperature for 22 hours, the resultant condensation cake was fragmented and acidified with 49 ml. of concentrated hydrochloric acid, added slowly. The resultant yellow suspension was refrigerated at 5° for 40 hours.

[1] Kindly supplied by Dr. James M. Sprague, Merck Sharp & Dohme, Rahway, N. J.

[2] All melting points were taken on a Fisher-Johns apparatus and are uncorrected.

Filtration, followed by washing with three 250-ml. portions of boiling ethanol gave 46.2 Gm. (76.6%) of microcrystals, m.p. 197–200° [Erlenmeyer (17) reported 192–193°, Shaw and Fox (8) reported 185–200°].

Calcd. for neut. equiv. (formol): 199. Found: 203.

Anhydrous Threo-β-phenylserine (9).—Forty-five grams of the monohydrate in 300 ml. of boiling water recrystallized as layered plates after dilution with 300 ml. of boiling methanol and refrigeration overnight. The plates were suction dried and placed in a vacuum desiccator at 50–60° and dried over calcium chloride for 3 hours. The product melted at 196–196.5° [lit. (17) m.p. 195–196°]. Calcd. for neut. equiv. (formol): 181. Found: 182, 183. R_f, 0.47, 0.46 [lit. (9) 0.43].

Preparation of Threo-β-phenylserine Methyl Ester Hydrochloride (18, 19).—Fifteen grams (0.085 mole) of the anhydrous threo acid was suspended in 150 ml. of absolute methanol in a 500-ml. three-neck flask equipped with a water condenser and drying tube, and the mixture was stirred magnetically. A steady stream of dry hydrogen chloride was passed into the suspension at a rate sufficient to promote gentle refluxing. The solid dissolved immediately, and after 2.5 hours the yellow solution had cooled to room temperature. The solution was filtered after standing overnight to remove any erythro isomer formed and evaporated to dryness to give a light yellow solid. The solid was dissolved in a minimum amount of hot methanol and crystallized by the addition of 6 volumes of anhydrous ether. Recrystallization was effected from methanol-ether (1:6) at −15° as glistening white flakes, m.p. 162–163.5° (decompn.), 13.4 Gm., (70.5%). Shaw and Fox (8) reported 160° (decompn.), Bergmann, *et al.* (18), 161° (decompn.), Carrara and Weibnauer (19), 156° (decompn.).

Erythro-β-phenylserine Amide.—Seven grams (0.036 mole) of the threo methyl ester was dissolved in 300 ml. of absolute ethanolic ammonia at 0° contained in a 1-L. thick-walled round-bottom flask. The flask was stoppered and wired shut in a cage while at 0° and allowed to stand at room temperature for 72 hours.

After standing, the flask was cooled to 0° and reopened. Evaporation of the solution under reduced pressure gave a yellow solid which was taken up in absolute ethanol, and the evaporation was repeated. This procedure was repeated twice more to remove all traces of ammonia. The yellow solid was recrystallized from hot absolute methanol yielding 2.2 Gm. (36%) of white fluffy needles, m.p. 191–193°.

Anal.—Calcd. for $C_9H_{12}N_2O_2$: N, 15.55. Found: N, 15.25, 15.27, 15.05.

Isolation of α-Aminocinnamamide from Ammonolysis Experiment.—A 15-Gm. quantity (0.08 mole) of the methyl ester was dissolved in 300 ml. of absolute methanolic ammonia at 0° and the flask was sealed as previously described. The solution was shaken continuously for 60 hours at room temperature. The flask was then cooled to 0° and opened. The excess ammonia and methanol were removed at reduced pressure to yield a yellow residue which dissolved in 75 ml. of hot methanol. Refrigeration of the solution at 5° for 3 hours gave 2.2 Gm. of a tan powder, m.p. 186–190°. Evaporation of the filtrate under reduced pressure to dryness gave a red solid which partially dissolved in 100 ml. of hot benzene. Refrigeration of the filtrate at 5° for 12 hours gave 1.5 Gm. of yellowish-white needles, m.p. 122-123°. An additional 1.5 Gm. of product was obtained by concentration of the filtrate and refrigeration.

Anal.—Calcd. for $C_9H_{10}N_2O$: N, 17.28. Found: N, 17.27, 17.36, 17.56.

N - Carbobenzoxy - threo - β - phenylserine Methyl Ester.—Five grams (0.022 moles) of the threo methyl ester hydrochloride was treated with an excess of potassium bicarbonate solution. The solution was extracted, portionwise, with 300 ml. of ethyl acetate. The ethyl acetate layers were dried over sodium sulfate and placed in a 1-L. 3-neck flask equipped with a stirrer and a drying tube. The solution was cooled in an ice-salt bath and treated with 5 Gm. of solid potassium bicarbonate. To the vigorously stirred solution, 3.0 Gm. of carbobenzoxy chloride was added in two portions. The cloudy mixture was stirred in the cold for 4 hours, at which time no more carbon dioxide was evolved. Fifteen milliliters of dry pyridine was added. The resulting mixture was alternately washed with 100-ml. portions of water, dilute hydrochloric acid, and water. The organic layer was dried over sodium sulfate and evaporated under reduced pressure to one-half the original volume. Refrigeration overnight gave a white slurry which yielded 2.0 Gm. of amorphous material when suction filtered and dried. Concentration of the filtrate and similar treatment gave an additional 2.5 Gm. Total yield was 73.5%. Crystallization from hot ethyl acetate gave white fluffy plates, m.p. 91.5–93°.

Anal.—Calcd. for $C_{18}H_{19}NO_3$: N, 4.26. Found: N, 4.64.

N - Carbobenzoxy - threo - β - Phenylserine Amide.—A 2.5-Gm. quantity (0.0076 mole) of the carbobenzoxy methyl ester was dissolved in 100 ml. of absolute methanolic ammonia according to previous procedures and allowed to stand at room temperature for 40 hours. Processing gave a white solid, weighing 2.1 Gm., m.p. 152–153° (88%). Recrystallization from methanol-water and vacuum drying gave fine white needles, m.p. 159–160°.

Anal.—Calcd. for $C_{17}H_{18}N_2O_4$: C, 64.96; H, 5.73; N, 8.91. Found: C, 64.35; H, 5.84; N 8.55.

Preparation of the Threo-amide.—Two grams (0.0063 moles) of the carbobenzoxyamide was dissolved in 100 ml. of methanol in a 300-ml. long-neck round-bottom flask, palladium catalyst was added, and the flask was fitted with a gas delivery adapter. The mixture was then shaken continuously while being flushed with a stream of dry nitrogen for 10 minutes. The amide was then reduced with a constant stream of hydrogen until the exit gases failed to precipitate barium carbonate in a saturated solution of barium hydroxide. The flask was again flushed with dry nitrogen for 10 minutes. The catalyst was removed by filtration through a Celite mat, washed with three small portions of methanol, and the filtrate was evaporated under reduced pressure to a semisolid mass. Drying in a vacuum desiccator over calcium chloride gave 1.1 Gm. of solid material (90.5%). The solid was washed with 10 ml. of ice-cold ether and recrystallized from methanol-petroleum benzin (cold). The final product was a white microcrystalline solid, m.p. 144–145°.

Anal.—Calcd. for $C_9H_{12}N_2O_2$: C, 60.00; H,

6.66; N, 15.55. *Found:* C, 60.54; H, 6.46; N, 15.34.

Phenyl(2 - hydroxy - 3 - pyrazyl)carbinol Hydrochloride.—Five grams (0.028 mole) of the threo amide was placed in a 100-ml. 3-neck flask equipped with a stirrer and dropping funnel and suspended in 50 ml. of absolute methanol. The flask was immersed in a dry ice bath and the contents stirred until the internal temperature reached −20°. Seven grams (0.035 moles) of 30% glyoxal was added all at once and 6 ml. (0.07 mole) of sodium hydroxide (12 *N* solution) was added dropwise over 15 minutes.

The tan suspension was stirred for 3 hours at −20°, 2 hours at room temprature, then acidified with 7 ml. of concentrated hydrochloric acid at 15°. A tan material precipitated which redissolved upon the addition of 10 ml. of water. Refrigeration at −20° for 40 hours gave 2.2 Gm. of a tan powder (39.4%), m.p. 199–203° (decompn.). Washing with ice-cold ether and crystallization from warm ether gave a tan microcrystalline powder, m.p. 199–201° (decompn.). Recrystallization from ethanol (charcoal)-ether (cold) gave fluffy plates, m.p. 203–204.5° (decompn.).

Anal.—Calcd. for $C_{11}H_{11}ClN_2O_2$: C, 55.50; H, 4.63; N, 11.79. Found: C, 56.35; H, 5.06; N, 12.34.

Phenyl(2 - hydroxy - 5 - methyl - 3 - pyrazyl)carbinol.—Six grams (0.034 moles) of the threo amide was suspended in 35 ml. of absolute methanol in a manner similar to above and treated with 7.0 Gm. (0.032 mole) of pyruvic aldehyde added in three portions over a 5-minute period. An equimolar amount (amide) of 12 *N* sodium hydroxide was added in 10 minutes over a 15-minute period. The suspension was stirred at −20 to −30° for 4 hours then at room temperature for 1 hour. The solution was acidified with concentrated hydrochloric acid to pH 6.8, treated with a small amount of solid sodium bicarbonate, and stirred for 10 minutes. Suction filtration gave 5.5 Gm. of a tan residue. The residue was washed with three 50-ml. portions of ice water and crystallized from hot acetone to give 3.2 Gm. (46.5%) of fluffy white needles, m.p. 174–176° (decompn.).

Anal.—Calcd. for $C_{12}H_{12}N_2O_2$: C, 65.60; H, 5.56; N, 12.95. Found: C, 64.99; H, 5.99; N, 12.91.

Phenyl(2 - hydroxy - 5,6 - dimethyl - 3 - pyrazyl)carbinol.—A suspension of 3.6 Gm. (0.02 mole) of the threo amide in 40 ml. of absolute methanol was treated with 2.58 Gm. (0.03 mole) of butanedione at −20°. While stirring, 4.5 ml. of 12 *N* sodium hydroxide was added over 20 minutes. The mixture was stirred for 4 hours and then for 1 hour at 0°. The dark brown solution was then acidified with concentrated hydrochloric acid to pH 7, diluted with 5 ml. of water, and allowed to come to room temperature. Tarry material present was precipitated by the addition of 15 ml. of water and the filtrate refrigerated to yield a tan granular material. Crystallization from 20% aqueous methanol gave 1.3 Gm. (33%) of light tan flakes, m.p. 181.5–183° (decompn.).

Anal.—Calcd. for $C_{13}H_{14}N_2O_2$: C, 67.82; H, 6.09; N, 12.17. Found: C, 67.20; H, 6.05; N, 11.80.

Phenyl(2 - hydroxy - 5,6 - diphenyl - 3 - pyrazyl)carbinol.—To 5 Gm. (0.028 mole) of the threo amide, suspended in 50 ml. of absolute methanol contained in a 3-neck flask equipped with a stirrer, water condenser, and dropping funnel, was added 5.9 Gm. (0.028 mole) of benzil. The mixture was stirred and heated to reflux, after which 4.85 ml. of 12 *N* sodium hydroxide was added slowly. The solution was refluxed for 30 minutes, cooled to room temperature, and then acidified with concentrated hydrochloric acid, dropwise. One gram of solid potassium bicarbonate was added during a 10-minute stirring period. The suspension was cooled to 0° and filtered. The yellow residue was washed twice with 500 ml. of water. Air drying overnight gave 6.5 Gm. (65.8%) of yellow powder which recrystallized from hot butanol in yellow flakes, m.p. 213–216° (decompn.).

Anal.—Calcd. for $C_{23}H_{18}N_2O_2$: C, 77.96; H, 5.07. Found: C, 77.78; H, 5.41.

Methyl Ether of the Prepared Compounds.—The methyl ethers of the hydroxypyrazines were formed by treatment with dimethylsulfate (equimolar) in dilute aqueous base. Isolation procedures and analytical data for the compounds follow. All reactants were mixed at 0°, followed by reflux for at least 1 hour.

Phenyl(2-methoxy-3-pyrazyl)carbinol.—The reaction filtrate was cooled to room temperature and filtered to remove a crop of tan microcrystals. Refrigeration at 5° overnight gave an additional crop of crystals. From 0.5 Gm. of pyrazyl compound, 0.185 Gm. (34%) was obtained. Recrystallization from hot water gave fluffy tan microcrystals, m.p. 140–142°.

Anal.—Calcd. for $C_{12}H_{12}N_2O_2$: N, 12.96. Found: N, 12.53.

Phenyl(2 - methoxy - 5 - methyl - 3 - pyrazyl)carbinol.—Refrigeration of the reaction filtrate for 30 hours gave a precipitate of yellowish-white rods. Filtration and recrystallization from hot water gave 0.115 Gm. of white rhombs, m.p. 134.5–136.5°, from 2.0 Gm. of starting material.

Anal.—Calcd. for $C_{13}H_{14}N_2O_2$: N, 12.17. Found: N, 11.85.

Phenyl(2 - methoxy - 5,6 - dimethyl - 3 - pyrazyl)carbinol.—The reaction mixture from 0.4 Gm. of reactant was cooled to room temperature and filtered to give a soft gummy residue which was air-dried. The residue was dissolved in a minimum amount of hot acetone and cooled to 5° for 30 hours, giving 0.048 Gm. of tan flakes which were recrystallized from ether-petroleum benzin, m.p. 110–111.5°.

Anal.—Calcd. for $C_{14}H_{16}N_2O_2$: N, 11.47. Found: N, 11.08.

Phenyl(2 - methoxy - 5,6 - diphenyl - 3 - pyrazyl)carbinol.—When the reaction mixture from 2.0 Gm. of reactant was cooled to room temperature, a small amount of insoluble gum was filtered off. After 2 hours at room temperature, the cloudy solution was treated with methanol until clear, an equal volume of water was added, and the mixture was refrigerated at 5° for 10 hours. A greenish-yellow solid precipitated. Recrystallization from cold aqueous methanol gave 0.035 Gm. of a yellow amorphous solid, m.p. 94.5–96° (decompn.).

Anal.—Calcd. for $C_{24}H_{20}N_2O_2$: N, 7.60. Found: N, 7.48.

SUMMARY

1. A series of hydroxypyrazines, with a phenylcarbinol moiety in the 3-position, was prepared. The prepared compounds were unobtainable by any other existing method.

2. A study of β-phenylserine indicated that the amide of this acid is more stable in the threo form than in the erythro form. The erythro form is preferentially obtained by reaction mechanism due to the excess pressure required in the reaction. The inversion during the formation of the amide from the ester is minimized by the use of the bulky carbobenzoxy group.

3. It was shown, by infrared analysis and low yield of methoxy compound from reaction with dimethylsulfate, that the compounds exist in predominantly the keto or pyrazone form. In addition, it was shown that sufficient crowding of the 2-position is present to prevent normal hydroxylic and ketonic reactions due to preventing sterically the formation of the necessary transition states required for these compounds.

4. The steric crowding has little effect on the formation of the methyl ethers because of the size and the linearity of the entering group.

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Influence of Excipients on the Analysis of Tablets and Capsules by Nonaqueous Titrimetry

By L. G. CHATTEN and C. A. MAINVILLE

Investigation of the effect of twenty-seven tablet and capsule excipients on the titration of medicinal organic acids and bases has shown that a careful choice of solvents can limit interference to a very small percentage of the excipients. Certain of these excipients which are titratable when dissolved alone in common nonaqueous solvents do not always consume titrant when combined with strong organic acids and bases. Polyvinylpyrrolidone and stearic acids cause the most difficulty in the titration of medicinal organic bases and acids, respectively.

ALTHOUGH the scientific literature of the past decade is filled with reports on the application of nonaqueous titrimetry to the analysis of drugs and chemicals, only a relatively few workers, by comparison, have adapted this technique to the analysis of pharmaceuticals. This situation can be attributed in part to the extensive use made of glacial acetic acid as a solvent, both by numerous investigators in the field as well as by such authoritative compendia as the British and United States pharmacopoeias (1, 2). While the wide solubilizing properties of glacial acetic acid enhance its utility, it is this very factor that limits its application in the analyses of pharmaceuticals. A second contributing cause to the reluctance of some workers in the field to accept nonaqueous titrations as a practical means of assaying pharmaceuticals has been the lack of investigations into the effect of excipients on the quantitative determination of active components of pharmaceutical forms.

The purpose of this report is to assess the influence of a number of excipients and to investigate means of obviating interference by altering the solvent system. The effect of the presence of drugs on interfering excipients is also within the scope of this work.

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EXPERIMENTAL

Apparatus

A microburet graduated to 0.01 ml.; electromagnetic stirrers; a precision Shell titrometer fitted with Beckman glass electrode No. 1190–80, and Beckman sleeve-type calomel electrode No. 1170–71.

Reagents

Perchloric acid in dioxane 0.05 *N;* potassium hydroxide in anhydrous methanol 0.1 *N;* and 6% mercuric acetate in glacial acetic acid. Solvents: acetone, A.C.S.; acetonitrile A.C.S. redistilled at 81.6° prior to use; benzene A.C.S.; chlorobenzene A.C.S., redistilled at 131–132° prior to use; chloroform A.C.S.; dimethylformamide, practical, redistilled at 150° prior to use; ethylene glycol A.C.S.; technical *n*-hexane; isopropanol A.C.S.; anhydrous methanol A.C.S.; practical nitromethane redistilled at 101.2°; propylene glycol A.C.S.

Indicators

Bromocresol purple 0.5% in anhydrous methanol; crystal violet 0.5% in glacial acetic acid; methyl red 0.1% in glacial acetic acid; methyl red 0.5% in anhydrous methanol; methylene blue 0.1% and quinaldine red 0.2% in anhydrous methanol; thymol blue 0.5% in anhydrous methanol.

Excipients

Acacia, 1; beeswax, 2; calcium carbonate, 3; calcium phosphate, 4; calcium phosphate tribasic, 5; calcium sulfate, 6; carnauba wax, 7; cetyl alcohol, 8; gelatin, 9; lactose, 10; magnesium carbonate, 11; magnesium hydroxide, 12; magnesium stearate, 13; magnesium sulfate, 14; methylcellulose, 15; polyethylene glycol 4000, 16; polyvinyl pyrrolidone, 17; sodium alginate, 18; sodium benzoate, 19; sodium carboxymethylcellulose, 20; sodium silicate, 21; sodium sulfate, 22; starch, potato, 23; stearic acid, 24; sucrose, 25; talcum, 26; tragacanth, 27.

PROCEDURE

General.—An excess amount of excipient was placed in a 150-ml. beaker, 50 ml. of solvent added, and stirring was then carried out for 15 to 20 minutes. The undissolved excipient was removed by filtration through a fine sintered-glass funnel. The appropriate indicator was added, and the filtrate was then titrated with 0.05 *N* perchloric acid in dioxane in order to determine those excipients which consume this titrant. A second set of filtrates was titrated with 0.1 *N* potassium hydroxide in methanol in the presence of the indicator of choice, in order to determine which excipients behave as acids.

All titrations with potassium hydroxide in methanol were performed in a closed system to prevent contamination by atmosphere. A blank was carried out on each solvent system and in all cases was found to be within reasonable limits.

Special procedures.—In the phenol-chloroform-acetonitrile system, a stock solution of 25% phenol in chloroform was prepared. The excipients were stirred with 25 ml. of this solution for the prescribed time, filtration was carried out and 25 ml. of acetonitrile was added to facilitate the visual titration.

In the glycols-isopropanol systems (25:25), because these solvent systems were viscous, filtration was carried out through a sintered-glass funnel of medium porosity. Using *n*-hexane, excipients were dissolved in 30 ml. of hexane, filtration was carried out, and 20 ml. of acetone added to facilitate the titration.

Testing the Effect of Interfering Excipients in the Presence of Drugs.—A quantity of drug sufficient to give a 3 to 4 ml. titration was accurately weighed and dissolved in the solvent of choice by stirring electromagnetically for 15 minutes. The appropriate indicator was added and the titration performed with 0.05 *N* perchloric acid in dioxane for bases. In the presence of a halide salt, 2 ml. of mercuric acetate solution was added to the titration. Organic acids were titrated by 0.1 *N* potassium hydroxide in methanol. All assays were carried out in duplicate. In certain instances, the visual end points was verified potentiometrically.

A similar quantity of active ingredients was accurately weighed and dissolved in 50 ml. of the solvent of choice. Varying amounts of excipients were added. The solution was stirred for 15 minutes and then filtered through a sintered-glass funnel of medium porosity to remove undissolved excipients. Two ml. of mercuric acetate solution was used when necessary. The indicator of choice was added and the titration performed with the appropriate titrant. Blanks were subtracted.

For perchloric acid titrations, 0.1% methyl red in glacial acetic acid was used as the indicator in every instance except for isopropanol where 0.5% thymol blue in methanol was used.

When potassium hydroxide was the titrant, 0.5% bromothymol blue was the indicator of choice for the solvent anhydrous methanol. For all other solvents, 0.5% thymol blue in methanol was the most suitable indicator.

TABLE I.—EXCIPIENTS TITRATABLE WITH 0.05 *N* PERCHLORIC ACID

Solvent	Indicator	Interfering Excipients
Acetone	Methyl red (CH_3COOH)	17, 19, 23
Acetonitrile	Methyl red (CH_3COOH)	17, 19
Benzene	Crystal violet	16, 17
Chlorobenzene	Crystal violet	16, 17
Ethylene glycol	Methyl red (CH_3OH)	13, 19, 21
n-Hexane	Methyl red (CH_3COOH)	None
Isopropyl alcohol	Methyl red (CH_3COOH)	17, 19
Methanol	Thymol blue	12, 13, 14, 17, 18, 19, 20, 21, 22, 27
Nitromethane	Methylene blue & quinaldine red	16, 17, 18, 19, 20, 21, 22
Phenol-chloroform-acetonitrile	Methyl red (CH_3COOH)	15, 19
Propylene glycol	Thymol blue	12, 13, 14, 17, 18, 19, 21, 22

RESULTS AND DISCUSSION

Although suitable indicators were known for some of the solvent systems, it was necessary to find indicators for others. This was accomplished by checking the color changes against a potentiometer which was equipped with a glass-calomel electrode system.

Twenty-seven excipients which occur most commonly in tablets and capsules were titrated in the manner outlined under Experimental, and those which consumed the amount of titrant in excess of the blank are reported in Tables I and II. Titration of these same excipients in chloroform and glacial acetic acid have been reported in a previous publication (3).

Table I shows that acetone, acetonitrile, benzene, chlorobenzene, *n*-hexane, isopropanol, and phenol-chloroform-acetonitrile are highly satisfactory solvents for the titration of medicinal compounds in tablets and capsules by perchloric acid, as very few excipients consume titrant in these solvents. Interference by polyvinylpyrrolidone and sodium benzoate seems to be widespread. It was found that in *n*-hexane no excipient consumed titrant. The utility of this solvent will be investigated and results published in a later report.

Table II shows that acetone, acetonitrile, benzene, chlorobenzene, and *n*-hexane are highly desirable solvents for the titration of acidic medicinal agents which are found in pharmaceuticals. Beeswax, polyvinylpyrrolidone, and stearic acid, however, are the common interfering agents.

From the results reported here, it is apparent that a careful choice of solvent system can markedly reduce and may even virtually eliminate interference by excipients in the nonaqueous titration of tablets and capsules.

The recoveries of the organic bases and acids are given in Tables III and IV, respectively. It can be seen from these tables that interfering excipients do not always cause overestimation when in the presence of some drugs and in certain solvents. It must be remembered that excipients were present in an amount far in excess of their normal occurrence in tablets. This produced, in certain instances, end points which were difficult to detect because they were seriously obscured by the formation of precipitates.

TABLE II.—EXCIPIENTS TITRATABLE WITH 0.1*N* POTASSIUM HYDROXIDE

Solvent	Indicator	Interfering Excipients
Acetone	Thymol blue	2, 19, 24
Acetonitrile	Thymol blue	17, 24
Benzene	Thymol blue	2, 24
Chlorobenzene	Thymol blue	2, 24
Chloroform	Thymol blue	2, 13, 17, 24
Dimethylformamide	Thymol blue	2, 7, 13, 14, 17, 19, 24
Ethylene glycol	Methyl red (CH_3OH)	24
Hexane	Thymol blue	2, 24
Isopropyl alcohol	Thymol blue	2, 17, 24
Methanol	Bromocresol purple	17, 24
Propylene glycol	Bromocresol purple	17, 18, 24

TABLE III.—TITRATION OF DRUGS AND EXCIPIENTS WITH PERCHLORIC ACID IN DIOXANE

Solvent	Active Ingredient	Excipient	Recovery, %
Acetone	Diphenhydramine HCl	...	100.5[a]
	Diphenhydramine HCl	Polyvinylpyrrolidone	109.7
	Diphenhydramine HCl	Sodium benzoate	100.8
	Diphenhydramine HCl	Starch	99.3
	Naphazoline	...	98.6[a]
	Naphazoline	Polyvinylpyrrolidone	109.2
	Naphazoline	Sodium benzoate	99.4
	Naphazoline	Starch	97.8
Acetonitrile	Diphenhydramine HCl	...	100.4
	Diphenhydramine HCl	Polyvinylpyrrolidone	99.7
	Diphenhydramine HCl	Sodium benzoate	102.7
	Naphazoline	...	98.6
	Naphazoline	Polyvinylpyrrolidone	99.2
	Naphazoline	Sodium benzoate	99.4
Benzene	Naphazoline	...	99.1
	Naphazoline	PEG 4000	98.6
	Naphazoline	Polyvinylpyrrolidone	97.6
	Methoxamine	...	99.3
	Methoxamine	PEG 4000	99.4
	Methoxamine	Polyvinylpyrrolidone	98.0
Isopropyl alcohol	Diphenhydramine HCl	...	98.9
	Diphenhydramine HCl	Polyvinylpyrrolidone	100.8
	Diphenhydramine HCl	Sodium benzoate	209.0
	Methoxamine	...	98.2
	Methoxamine	Polyvinylpyrrolidone	99.3
	Methoxamine	Sodium benzoate	145.6
Phenol-chloroform-acetonitrile	Diphenhydramine HCl	...	100.2
	Diphenhydramine HCl	Methylcellulose	103.4
	Diphenhydramine HCl	Sodium benzoate	193.6
	Ephedrine HCl	...	99.5
	Ephedrine HCl	Methylcellulose	100.8
	Ephedrine HCl	Sodium benzoate	206.5

[a] Potentiometric determination.

TABLE IV.—TITRATION OF DRUGS AND EXCIPIENTS WITH POTASSIUM HYDROXIDE IN METHANOL

Solvent	Active Ingredient	Excipient	Recovery, %
Acetone	Acetylsalicylic acid	...	100.1
	Acetylsalicylic acid	Beeswax	101.1
	Acetylsalicylic acid	Sodium benzoate	99.8
	Phenobarbital	...	99.3
	Phenobarbital	Beeswax	100.8
	Phenobarbital	Sodium benzoate	99.8
Acetonitrile	Acetylsalicylic acid	...	99.8
	Acetylsalicylic acid	Cetyl alcohol	100.1
	Acetylsalicylic acid	Polyvinylpyrrolidone	100.2
	Phenobarbital	...	99.6
	Phenobarbital	Cetyl alcohol	99.7
	Phenobarbital	Polyvinylpyrrolidone	97.8
Chloroform	Acetylsalicylic acid	...	100.3
	Acetylsalicylic acid	Beeswax	105.2
	Acetylsalicylic acid	Magnesium stearate	111.1
	Acetylsalicylic acid	Polyvinylpyrrolidone	100.6
	Phenobarbital	...	99.7
	Phenobarbital	Beeswax	104.9
	Phenobarbital	Magnesium stearate	99.9
	Phenobarbital	Polyvinylpyrrolidone	100.8
Isopropyl alcohol	Acetylsalicylic acid	...	99.7
	Acetylsalicylic acid	Beeswax	100.2
	Acetylsalicylic acid	Polyvinylpyrrolidone	100.2
	Phenobarbital	...	99.8
	Phenobarbital	Beeswax	100.6
	Phenobarbital	Polyvinylpyrrolidone	100.4
Methanol	Acetylsalicylic acid	...	99.3
	Acetylsalicylic acid	Polyvinylpyrrolidone	99.9
	Benzoic acid	...	100.1
	Benzoic acid	Polyvinylpyrrolidone	100.8

As predicted, stearic acid readily consumed titrant in the presence of other organic acids and hence created a gross error in every solvent system. Beeswax, polyvinylpyrrolidone, and sodium benzoate were also major interfering agents but did not always consume titrant.

CONCLUSION

It has long been the feeling by some critics that the effect which excipients might exert on the quantitative analysis of tablets and capsules by nonaqueous titrimetry is probably so great as to limit its usefulness to the assay of the basic materials alone, or at best, to the products of those manufacturers where the control laboratory might be able to exert its influence in the formulation. The findings reported herein show that the careful choice of solvent systems can greatly minimize or even eliminate the possibility of interference by excipients.

It has been shown also that the nonaqueous titration of organic medicinal acids and bases suffers from very limited interference by tablet excipients, even when the excipients themselves are titratable. Sodium benzoate and polyvinylpyrrolidone appear to cause the major concern in the titration of bases. Only stearic acid causes serious and consistent interference in the titration of acids. This could be overcome by pharmaceutical manufacturers using magnesium stearate as a lubricant.

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Polymorphism and Drug Availability

Solubility Relationships in the Methylprednisolone System

By W. I. HIGUCHI†, P. K. LAU, T. HIGUCHI, and J. W. SHELL

The thermodynamic relationships involving polymorphism and solubility were examined in some detail and applied to experimental results with the methylprednisolone system. The solubilities of the two crystal forms of this steroid were determined in water, decyl alcohol, and dodecyl alcohol at various temperatures. The solubility ratios for the two forms were generally found to be independent of the solvent in accordance with theory. At room temperatures the activity of the more energetic form of the drug was found to be the order of 80% greater than that of the more stable form. The heat, entropy, and temperature of transition were calculated from the data, and the possible molecular factors involved in polymorphism were considered. Further work of exploratory nature involving cloudpoint determinations indicate that much more energetic crystal forms are probable.

AMONG THE numerous ways (1) of altering drug availability to the patient, methods involving changing the crystal form of the drug appear to have received proportionately the least detailed examination. Thus it appeared to us that an investigation of this problem would be worthwhile. This report represents the initial phase of a program aimed toward the understanding of factors determining activity, physical stability, and frequency of occurrence (or preparation) of polymorphs.

THERMODYNAMIC THEORY

For simplicity let us assume that a particular drug exhibits only two crystalline forms depending on the conditions of preparation. Let these be form I and form II, the former designating the more thermodynamically stable one at room temperatures.

We may then write

$$F_I^\circ = H_I^\circ - T^\circ S_I \qquad \text{(Eq. 1a)}$$

and

$$F_{II}^\circ = H_{II}^\circ - TS_{II}^\circ \qquad \text{(Eq. 1b)}$$

where F°, H°, and S° are the usual standard state Gibbs free energy, enthalpy, and entropy, respectively, for the two forms, and T is the absolute temperature in °K. For completeness we may also write the corresponding equation for the supercooled liquid

$$F_I^\circ = H_I^\circ - TS_I^\circ \qquad \text{(Eq. 1c)}$$

If now f_I and f_{II} are the drug fugacities (2) or escaping tendencies for the two phases in their standard states, we have then

$$RT \ln f_I/f_{II} = \Delta F_{I.II} = F_I^\circ - F_{II}^\circ \qquad \text{(Eq. 2)}$$

where R is the gas constant. Since fugacities may be equated to the vapor pressures in the limit of low pressures (gas ideality) and since vapor imperfection is generally negligible in our system, we may regard the fugacity ratio expressed in Eq. 2 as simply the vapor pressure ratio for the two forms.

By combining Eq. 1 and Eq. 2 we obtain the following relation

$$RT \ln f_I/f_{II} = \Delta F_{I.II}^\circ = \Delta H_{I.II}^\circ - T\Delta S_{I.II}^\circ \qquad \text{(Eq. 3)}$$

which relates the fugacities with the standard entropy, enthalpy, and free energy changes for the I to II phase transformation. Equation 3 is particularly useful in discussing the relationship between molecular arrangements in the crystals and fugacity of polymorphs.

As our interests are associated with drug availability with respect to solid-solution behavior we must introduce solubility into the theory. Adopting the convention that the activity becomes equal to molality at infinite solute dilution we can write (3)

$$\frac{\partial \ln (\gamma m)}{\partial T} = \frac{H^\infty - H^S}{RT^2} \qquad \text{(Eq. 4)}$$

Here m is the solubility in molal units, γ is the activity coefficient defined by

$$\gamma \to 1 \text{ as } m \to 0$$

H^∞ is the partial molar enthalpy of the solute at infinite dilution and H^S is the mola' enthalpy of the crystal. The difference

$$\Delta H = H^\infty - H^S$$

is the heat of dissolution at infinite dilution per mole of crystal.

Now when Henry's law is obeyed, *i.e.*, for dilute solutions in which solute association is negligible, solute fugacity of the solution will be proportional to the solute concentration. Thus under these conditions $\gamma = 1$ and $f_I/f_{II} = m_I/m_{II}$. Since most systems of interest to us involve dilute solutions, this case is of practical importance. Assuming Henry's law we may write from Eq. 4

$$\frac{\partial \ln m_I}{\partial T} = \frac{H^\infty - H_I^\circ}{RT^2} = \frac{\Delta H_I^\circ}{RT^2} \qquad \text{(Eq. 5a)}$$

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and

$$\frac{\partial \ln m_{II}}{\partial T} = \frac{H^{\infty} - H_{II}^{\circ}}{RT^2} = \frac{\Delta H_{II}^{\circ}}{RT^2} \quad \text{(Eq. 5b)}$$

for each of the two forms in their standard states. Here the ΔH°'s are the heats of solution at infinite dilution.

Equations 5a and 5b may be combined to give

$$\frac{\partial \ln (m_I/m_{II})}{\partial T} = \frac{\Delta H_{I,II}^{\circ}}{RT^2} \quad \text{(Eq. 6)}$$

Equation 6 relates the solubility (or fugacity) ratio to the enthalpy of phase change. It can be seen that

$$\frac{\partial \ln (m_I/m_{II})}{\partial T}$$

should be independent of the solvent as long as Henry's law is obeyed.

At some temperature, T_t, known as the transition temperature the fugacities of the two forms will be equal, *i.e.*, $f_I = f_{II}$ or $m_I = m_{II}$. From Eq. 3 we have the condition

$$\Delta H_{I,II}^{\circ} = T_t \Delta S_{I,II}^{\circ} \quad \text{(Eq. 7)}$$

at $T = T_t$.

It is apparent that the above thermodynamic relationships, if judiciously applied, should be helpful in the characterization and the evaluation of polymorphic systems. In particular, it is noteworthy that by means of solubility measurements alone it is possible to obtain the enthalpy and entropy differences between polymorphic forms of crystals. These thermodynamic quantities are useful in the understanding of the inter- and intramolecular crystalline factors determining the frequencies of occurrence and differences in activities among polymorphs.

EXPERIMENTAL

General Considerations.—Methylprednisolone was selected for this study because only two crystalline forms of this compound are known to exist. Furthermore, these can be easily prepared and characterized by X-ray or infrared methods.

Polymorph I was prepared by recrystallization from acetone and polymorph II by sublimation at 190°. Table I gives the X-ray powder diffraction data for the two forms. The infrared mull spectra (mineral oil) are presented in Fig. 1. Either of these two methods serve to characterize the two forms.

TABLE I.—METHYLPREDNISOLONE, INTERPLANAR SPACINGS, A

Polymorph I		Polymorph II	
9.87	2.81	12.10	3.32
9.21	2.70	8.58	3.26
8.42	2.51	7.37	3.16
7.08	2.44	6.32	3.13
5.94	2.37	6.06	3.10
5.50	2.32	5.79	3.04
5.01	2.25	5.21	2.93
4.59	2.16	5.09	2.89
4.19	2.13	4.71	2.86
3.93	2.08	4.57	2.78
3.67	2.06	4.52	2.72
3.51	1.99	4.27	2.58
3.39	1.92	4.08	2.55
3.29	1.84	3.98	2.43
3.11	1.79	3.88	2.37
2.96	...	3.72	2.34
2.91	...	3.67	...
2.85	...	3.38	...

To permit a test of the solvent independence of f_I/f_{II} (or m_I/m_{II}), three solvents—water, decyl alcohol, and dodecyl alcohol—were selected. The solubilities of methylprednisolone in these solvents are all low enough so that Henry's law is likely to be obeyed by these solutions.

Procedure.—For determinations of solubilities in

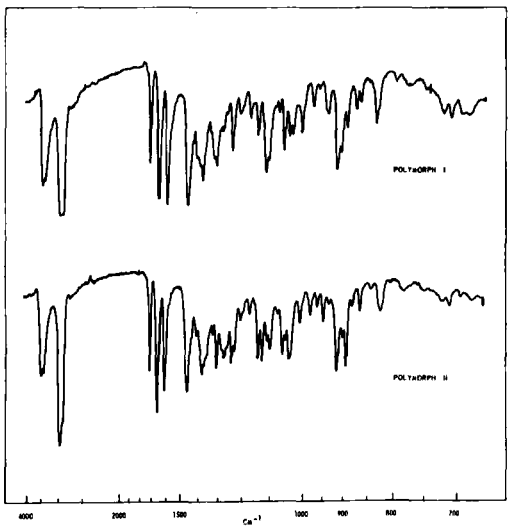

Fig. 1.—Infrared mull spectra of methylprednisolone, polymorphs I and II.

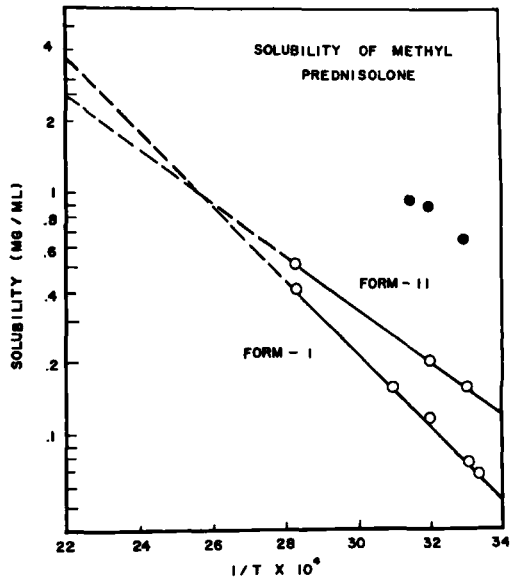

Fig. 2.—Water solubilities of methylprednisolone as a function of temperature: ○ refers to data for form I and Form II; ● is cloudpoint data (see text).

water, 15-ml. vials containing water and excess amounts of the particular form were slowly rotated in a water bath thermostated to ±0.05°. Periodically 5-ml. aliquots of the solution were withdrawn with a pipet fitted with a glass wool plug, diluted with 95% ethanol, and assayed spectrophotometrically. After several days the steroid concentrations in the solution phase were found to be constant.

For determinations in the alcohols, excess amounts of the particular form of the drug were placed with the solvent in a stoppered flask which was water jacketed to maintain a constant temperature within ±0.05°. The mixture was vigorously agitated with a magnetic stirrer. Aliquots of the solution were withdrawn and filtered by a syringe fitted with a hypodermic adapter for Millipore[1] filter paper. Then the solutions were diluted appropriately in 95% ethanol and assayed spectrophotometrically.

In the water system with both forms I and II the steroid concentration always increased with time to a constant value. This was also the general behavior with the alcohol systems at the lower temperatures. However, beyond 50° in the decyl alcohol case involving form II, the steroid concentrations initially increased with time, then reached a maximum value, and then decreased steadily. This can be explained on the basis of relatively rapid conversion of the drug to another form at the higher temperatures in decyl alcohol. Thus for form II in decyl alcohol, data were obtained only up to 60°.

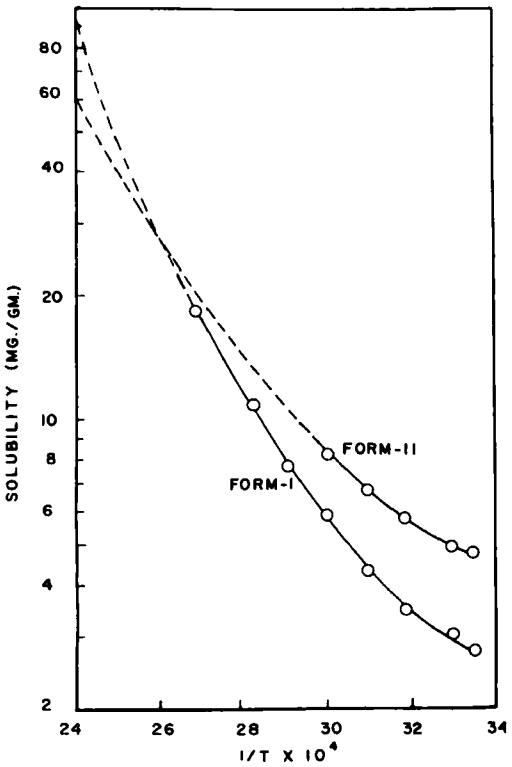

Fig. 3.—Solubility of the two forms of methylprednisolone in decyl alcohol as a function of temperature.

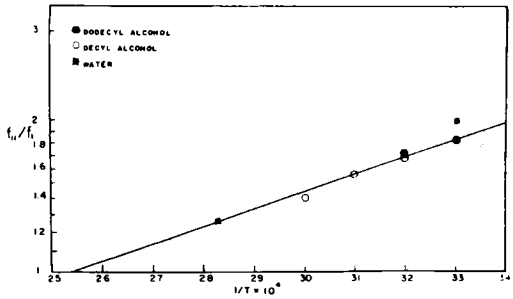

Fig. 4.—A plot of the fugacity or solubility ratio for the two forms of methylprednisolone as a function of solvent and temperature.

RESULTS AND DISCUSSION

The results of the determinations in water and decyl alcohol are shown in Figs. 2 (open circles) and 3, respectively. The data are plotted as log solubility *vs.* $1/T$ rather than *vs.* T. This method of plotting follows from rearranging Eq. 5 to give

$$\frac{\partial \ln m_I}{\partial (1/T)} = \frac{-\Delta H_I^\circ}{R} \qquad \text{(Eq. 8a)}$$

and

$$\frac{\partial \ln m_{II}}{\partial (1/T)} = \frac{-\Delta H_{II}^\circ}{R} \qquad \text{(Eq. 8b)}$$

In Fig. 4, these data and the data for the dodecyl alcohol system are all plotted as log solubility ratio *vs.* $1/T$. This type of plot is suggested by Eq. 6. Except for the results at the lowest temperatures, the solubility ratios appear to be independent of the solvent in accordance with theory. While it appears unlikely, the deviations at the low temperatures are possibly attributable to the deviations from Henry's law.

From the slope of the best straight line drawn through the points in Fig. 4 we may calculate a value for $\Delta H_{I,II}^\circ$. The transition temperature, T_t, may be obtained by extrapolating the curve to

$$\frac{f_I}{f_{II}} = \frac{m_I}{m_{II}} = 1$$

For these quantities we obtain $\Delta H_{I,II}^\circ = 1600$ cal. mole^{-1} and $T_t = 118°$. Then by application of Eq. 7 we obtain $\Delta S_{I,II}^\circ = 4.1$ e.u.

Paucity of data in the literature on entropies of transition and the lack of structural information on the methylprednisolone system preclude a single interpretation of the $\Delta S_{I,II}^\circ$ value in terms of molecular arrangement in the crystals. It appears likely, however, that the entropy difference is a result of greater localization of the functional groups in the side chain in form I resulting either from intermolecular or intramolecular interactions. It is well known (4) that lack of freedom of rotation about the carbon-carbon bond contributes about $R\ln 3$ per methylene group to the entropy of fusion for long chain paraffins. Thus the effects of two or more linkages in the side chain may account for the observed $\Delta S^\circ_{I,II}$.

[1] Millipore Filter Corporation, Bedford, Mass.

These results with form I and form II of methylprednisolone demonstrate that significant differences in solubilities may exist among polymorphs. In the present example the unstable form, II, exhibits a solubility the order of 80% greater than that for the more stable form, I, at room temperature. In order to obtain an idea as to the order of magnitude of the solubility of amorphous methylprednisolone, exploratory studies[2] involving cloudpoint determinations were carried out. The results of these are presented in Fig. 2 (darkened circles). These "solubility" values represent the concentrations of the steroid in aqueous solutions necessary for rapid appearance of turbidity when a concentrated dimethylformamide solution of the steroid was added dropwise to water. If it can be assumed that appreciable supersaturation is unnecessary for nucleation of the supercooled liquid phase, it is reasonable to presume that these values represent a lower limit for the solubility of the supercooled liquid or the amorphous solid. It is noteworthy that these correspond to almost 20 times the solubility of form I. It is hoped that future studies on this problem would lead to preparable crystal forms exhibiting such large differences in solubilities.

[2] Unpublished work.

REFERENCES

(1) See *e.g.*, Wagner, J. G., THIS JOURNAL, 50, 359(1961).
(2) Lewis, G. N., and Randall, M., "Thermodynamics," McGraw-Hill Book Company, Inc., New York, 1923, p. 190.
(3) Guggenheim, E. A., "Thermodynamics," North-Holland Publishing Company, Amsterdam, 1957, p. 292.
(4) For a recent discussion, see Aranow, R. H., Witten, L., and Andrews, D. H., *J. Phys. Chem.*, 62, 812(1958).

X-Ray and Crystallographic Applications in Pharmaceutical Research IV

Modified Procedures for Molecular Weight Determinations

By JOHN W. SHELL†

The modified procedures described in this paper for the determination of molecular weights of crystalline organic compounds differ from conventional procedures in the replacement of film recording by proportional counter recording and by the use of fractional-cell, rather than true unit-cell volumes. These procedures allow the routine determination of molecular weights with relatively small investments of time, yet with errors usually no greater than one hydrogen atom. The general method is presented and detailed procedures for lower symmetry crystal systems, in which most organic compounds fall, are described. The procedures are illustrated by specific examples.

FOR THE precise determination of molecular weights of crystalline compounds, the X-ray diffraction method has long been the method of choice. If one invests sufficient time and all the necessary care, the accuracy of this method need be limited only by errors due to crystal imperfections, which are often of the order of 0.04% if due to void spaces and often less if due in part to inclusions.

The strong relationship between time investment and accuracy appears, for practical purposes, to have a point of diminishing returns. Experience has shown that somewhat less than absolute, but yet fairly accurate determinations can be made with reasonable time investments.

In these laboratories an attempt has been made to develop procedures which assure an acceptable level of accuracy (the error usually approximating one hydrogen atom) with a minimum investment of operator time. This has required differing procedures for crystals of different symmetry, as noted below. It has also required the replacement of film techniques with direct recorder techniques.

GENERAL METHOD

The feasibility of the X-ray method for molecular weight determinations derives from the fact that an exact and determinable numbe; of molecules occupies a *unit cell* which, by three-dimensional repetition, generates the space lattice of the crystal. As the repeat spacings are of the same order of magnitude as X-ray wavelengths, diffraction of X-rays is possible and oriented diffraction procedures permit the measurement of the unit cell volume. An experimentally determined unit-cell density (crystal density) permits then the simple calculation of the unit-cell weight. This is either the molecular weight or some

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multiple of it, depending upon the number of molecules per cell. The number of molecules per cell is determinable.

The conventional approach to the measurement of the unit-cell volume has been by the use of X-ray film cameras and oriented crystal procedures producing values for the true cell edge lengths and the angular relationship between the true cell edges. A modified approach is by use of proportional counter detection and the measurement of fractional cells, rather than true cells, resulting in a more rapid accumulation of accurate information.

In the sections which follow, two crystal density determination methods are described, followed by modified procedures for the determination of unit-cell volumes for the various crystal symmetries encountered most often with organic compounds. The methods are illustrated by specific examples.

Determination of the Crystal Density

Two methods of crystal density determination have been found to be highly reliable. The first, which is more rapid, is less accurate and tends to limit the molecular weight determination to the density error, usually about 2×10^{-3} Gm./ml. The second method requires more time but allows the accuracy of the molecular weight determination to be limited to either that of the X-ray measurements or to the crystal imperfections.

Method 1.—This less accurate, but more rapid method is an adaptation of the flotation method of Bernal and Crowfoot (1), and consists essentially of immersing a few crystals in a light liquid, followed by addition of a miscible heavy liquid until the crystals tend to rise in the mixture. At this point the heavy liquid is added dropwise to the mixture with stirring. Each addition is followed by a short period of centrifugation. This is continued until complete dispersion is maintained even after prolonged centrifugation. The temperature of the suspension is noted, using the same thermometer which is later to be used as part of a standard pycnometer, and the suspension cooled to a few degrees below this temperature. The suspension is then used to fill the pycnometer and the volume adjusted when the temperature reaches the original suspension temperature. The density is then computed from the weight of the known volume.

The centrifuge should be of the clinical type, which permits the tubes to swing to a horizontal position. A convenient volume to use is 10 to 12 ml. so that standard centrifuge tubes and a 10-ml. pycnometer may be used.

When only a few crystals are available, it is convenient to do all mixing in a small (25-ml.) beaker, adding each new liquid mixture to the few crystals in a 3-ml. test tube for centrifuging. If, in the early stages of the determination, density equilibration is approached by adding the heavy liquid to the light liquid, centrifugation after each addition will pack the few crystals to the bottom of the tube and the liquid can be poured into the breaker for the next addition and mixing. This confines the crystals to the small volume of the tube and conserves them even during a large number of liquid additions and a large total volume increase.

The liquids used for flotation must obviously be nonsolvents for the crystals of interest. Both an aqueous system and a series of organic liquids have been used with good success, depending upon the solubility of the crystals. The aqueous system is comprised of various concentrations of zinc chloride which allow increases in density from 1.0 up to about 2.0 and covers the density range of most organic compounds. The addition of a slight trace of Aerosol OT, or other wetting agent, to aqueous flotation liquids has been found to be advantageous. The organic series comprises the miscible liquids benzene (d = 0.879), α-chloronaphthalene (d = 1.194), α-bromonaphthalene (d = 1.487), s-tetrabromoethane (d = 2.964), and methylene iodide (d = 3.3). With either flotation system it has been found helpful to place the container with the lighter liquid and immersed crystals into some evacuable chamber so that the crystals may be freed from occluded air within a few minutes at reduced pressure.

During centrifugation, the flotation suspension undergoes a temperature rise; this is the source of the chief error of this method. For this reason, short centrifugation time and larger volumes are advantageous. A crystal density may be determined by this method in about 1 hour.

Method 2.—The most precise crystal density determination method is that of Straumanis. Since it has been previously described (2), it will receive only brief mention here.

Use is made of a special tube-shaped pycnometer constricted at the neck with a calibration mark in the area of constriction. A flotation liquid nearly equal in density to that of the suspended crystal is added past the mark and the filled pycnometer immersed in a water bath adapted for temperature control. The temperature is adjusted until suspension is achieved, at which time the volume is adjusted to the mark and the pycnometer and contents weighed. Knowing by previous calibration the pycnometer volume over a range of temperatures, the density of the crystal is calculated. The use of a cathetometer to observe the suspended crystals in the final stages of equilibration has been found advantageous. The accuracy of the method depends upon the difference in cubical expansion of the liquid and the crystal, which is usually quite large. The accuracy reported by Straumanis (2) was at least 3×10^{-4} Gm./ml. The time required for a determination is from 2 to 3 hours.

Measurement of the Unit Cell Volume and Calculation of the Molecular Weight

Many alloys, minerals, and other inorganic compounds crystallize in high symmetry systems. The measurement of their unit-cell dimensions can often be made directly from easily obtained powder-diffraction patterns. The organic compounds of pharmaceutical interest, however, almost invariably crystallize in lower symmetry systems and their powder-diffraction patterns are too complicated for the direct determination of unit cell dimensions. This is always true for triclinic and monoclinic crystals; indirect graphical methods have been extended with with some success, particularly to orthorhombic powder patterns, but only when each powder line is known to a high degree of accuracy, and accompanied by an extremely large investment of time. It has, therefore, become standard procedure to utilize single-crystal studies in place of the more easily acquired, but less meaningful, powder patterns.

Several types of single-crystal X-ray cameras are

well known. Most of them are designed for use in total structure determination, only the first step of which involves unit-cell measurements. Most all of these cameras use film for the detection of diffracted X-rays. The recently developed General Electric single crystal orienter, however, utilizes direct recording by a proportional or scintillation counter instead of film. Its use permits accurate measurements with a saving of much of the time otherwise required for the exposure, developing, drying, and measuring of the film for determination of each dimension. Moreover, unlike the procedures required in the use of most film cameras, all measurements may be made from a single mounting of the crystal. The attachment, mounted on a G. E. XRD-5 diffractometer, is shown in Fig. 1.

For unit-cell measurements, a single crystal is selected on the basis of size, shape and the freedom from small, adhering crystals. A polarizing microscope is ideal for the purpose of crystal selection. Crystal habit varies considerably within most crops of organic crystals. It is often convenient to select a crystal with an elongated prism axis. Compared to the optimum size for inorganic crystals (about 0.2 mm.), the low mass absorption coefficients of elements comprising organic compounds permits use of fairly large crystals (up to 1 mm., if properly positioned).

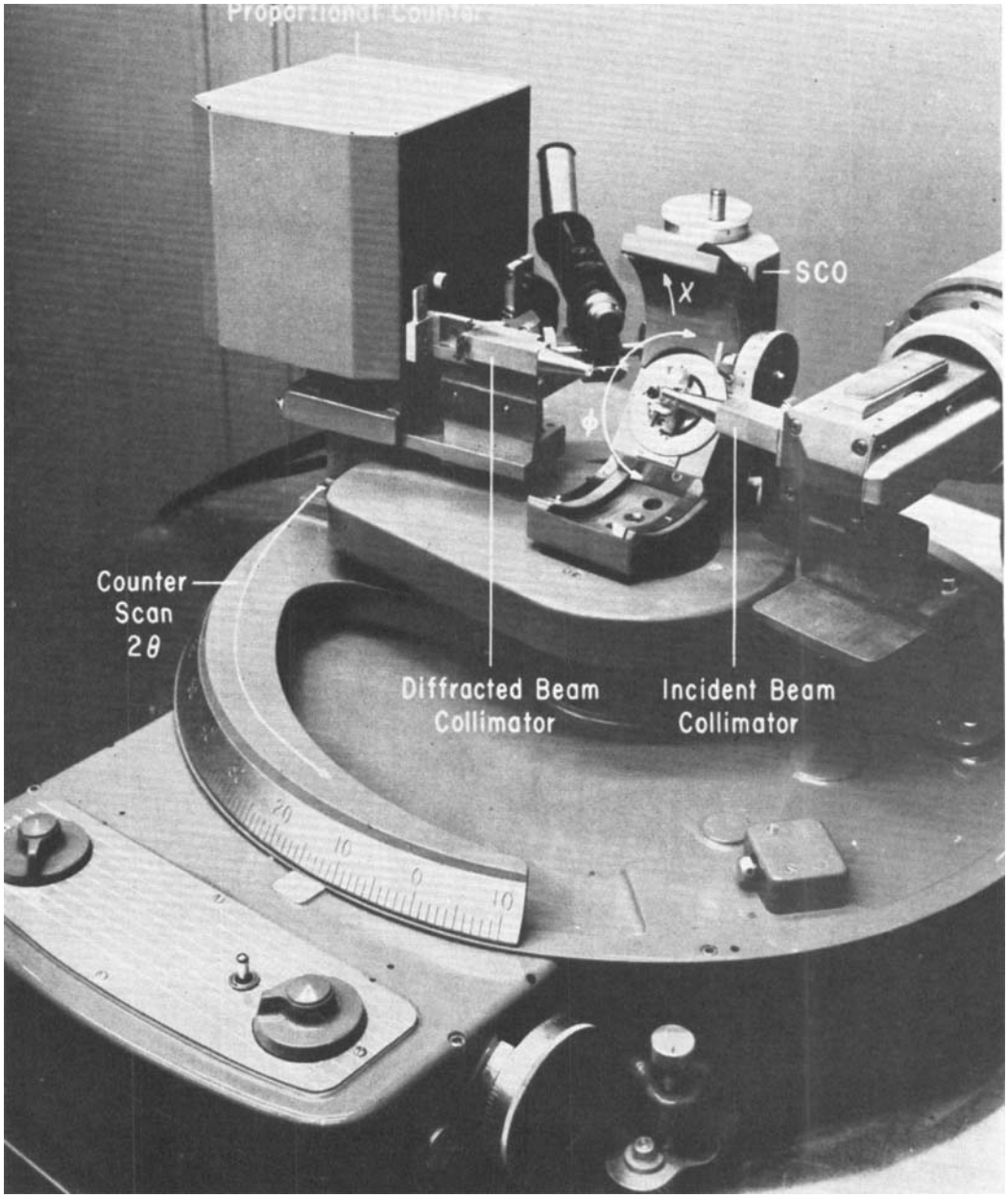

Fig. 1.—X-ray diffractometer and single-crystal orienter (SCO). Crystal rotation directions ϕ and χ are shown (radiation shields removed).

With the aid of a microscope, the crystal is cemented to the end of a fine glass fiber in such a manner that the fiber is approximately coincident with one of the crystallographic axes. The fiber is then attached to the goniometer head of the single crystal orienter (SCO) by the use of beeswax, plasticine, or other suitable medium. With the aid of the microscope shown in Fig. 1, the goniometer head permits orientation of the crystal at a point in space such that there is no departure of the crystal from this point, regardless of rotation about the various SCO axes. The principal SCO axes are phi (ϕ), about which the originally chosen crystal axis may be rotated, and chi (χ), about which this crystal axis may be inclined. The ϕ axis is rotatable through 360° and calibrated to 0.02°. The χ axis may be rotated from $-10°$ to $+100°$ and is calibrated to 0.01°. The ϕ axis is vertical when χ is set at 0°. All orientations of the crystal with respect to the X-ray beam are possible from a single mounting of the crystal. From a properly oriented crystal, the diffractometer measures the Bragg angle, 2θ, directly.

As the proportional counter shown in Fig. 1 scans in a counterclockwise direction, the SCO also rotates in this direction, but at half the angular velocity of the counter. The Bragg equation, $N\lambda = 2d \sin \theta$, relates the X-ray wavelength (λ), the diffraction angle between the diffracted and projected incident beams (2θ), and the crystal interplanar spacings (d), all for various orders of diffraction (N). Thus, when a diffraction "signal" is found at a certain 2θ position, solution of the Bragg equation gives the spacing within the crystal of parallel planes which are normal to the bisector of the angle between the incident and diffracted beams.

Since most pharmaceutically important compounds are organic, and most crystalline organic compounds are of either orthorhombic, monoclinic, or triclinic symmetry, these lower symmetry systems will be considered in detail. The higher symmetry systems, isometric, tetragonal, and hexagonal, can be treated as special, simplified cases of the lower symmetry systems described. For instance, a tetragonal crystal may be treated as an orthorhombic crystal which has two of its axes equal in length.

A. Crystals of Orthorhombic Symmetry.—Orthorhombic crystals are referred to three mutually perpendicular, unequal axes. The volume of an orthorhombic unit cell is simply the product of the three axial lengths or, in other words, the product of the dimensions of the repeat lattice in each of the three perpendicular directions.

The orthorhombic cell volume is measured by mounting the crystal with one of the crystallographic axes vertical, and coincident with ϕ, while $\chi = 0°$. With ϕ rotated until a major crystal face is parallel to the X-ray beam when $2\theta = 0°$, a scan of 2θ will generally give a first diffraction signal. Solution of the Bragg equation for the 2θ value thus obtained gives one of the cell dimensions or some submultiple of it. Repeating this operation after a rotation of 90° in ϕ produces the second cell dimension. The third cell dimension is obtained from a 2θ scan following a 90° rotation of χ. Orthorhombic axes are designated such that $c<a<b$.

The recording of 2θ data for each signal should be made only after the signal is maximized by slight adjustments of all three variables, χ, ϕ, and 2θ. Further, it should be noted that each diffraction position of the crystal may give rise to several signals at different 2θ values, each representing a different diffraction order. As certain errors diminish with increasing values of θ, higher order diffractions are advantageously used for the cell-dimension determination. Finally, this procedure sometimes results in a cell-dimension value which is one-half that of the true cell dimension, due to certain diffraction extinctions. A fractional, rather than true cell volume is thus obtained. For the purpose of molecular weight determination this is of no consequence as the error will result in only a different whole number value for the number of molecules determined per unit cell and not in a different value for the molecular weight itself.

When the crystal density and the unit-cell axes, a, b, c, (or possibly semiaxes) have been determined, the cell volume ($v = abc$ for orthorhombic crystals) and the molecular weight, M, of the compound are calculated (3)

$$M = \frac{dv}{1.6604\ n}$$

where d = density, v = cell volume, and, n = number of molecules per cell.

Although more involved methods exist for determining n, the number of molecules per cell, the simplest method which is invariably applicable consists of substituting for M in the above equation an approximate molecular weight value obtained from chemical evidence and solving for n. Knowing that the only possibilities for n in this crystal system are 1, 2, 4, 8, etc., the proper choice becomes obvious and the exact value of n may be used to calculate M.

Sometimes a significant gain in accuracy can be accomplished by the combined use of single-crystal data and powder-diffraction data. This can often be accomplished at an overall saving of time, since when this procedure is followed, the single-crystal data need not be so precise and can, therefore, be collected more rapidly.

The procedure involves rapid measurement of unit cell dimensions by the SCO operation described above and the use of these approximate values to partially index a powder diffraction pattern.[1] By a slow scan of only the three appropriate powder diffraction lines, using standard diffractometer techniques for high resolution,[2] the cell dimensions can be measured with extreme accuracy.

Examples of the results of the use of combined single-crystal and powder-diffraction data are shown

---

[1] For orthorhombic crystals,

$$d = \frac{1}{\sqrt{\frac{h^2}{a^2} + \frac{k^2}{b^2} + \frac{l^2}{c^2}}}$$

This reduces greatly for the determination of d for $h00$, $0k0$ and $00l$, usually found, with organic crystals, in the first few powder lines. Occasionally $hk0$, $h0l$, or $0kl$ will have to be calculated also, if one of the first series is absent.

[2] It has been found useful to mix a small amount of sodium chloride with the unknown material before measuring the powder diffraction pattern. The 2θ positions of all measured lines may then be corrected by the measured position of a sodium chloride line, and errors due to instrumental misalignment, temperature, etc. are compensated. Sodium chloride has a d spacing (100) of 5.64009Å. at 25°.

TABLE I.—RESULTS OF THE USE OF PRECISE POWDER DIFFRACTION DATA TO REFINE APPROXIMATE SINGLE-CRYSTAL DATA FOR UNIT-CELL MEASUREMENTS

	Crystal Density	Cell Dimensions, Å.		Molecular Weight					
				Approximate			Refined		
		Approximate	Refined	Found	True	% Error	Found	True	% Error
Example 1	1.255	a = 9.30	9.50						
		b = 11.01	11.00						
		c = 5.10	5.08	394.70	401.49	1.7	401.25	401.49	0.06
Example 2	1.197	a = 8.46	8.34						
		b = 12.86	12.93						
		c = 3.99	3.99	312.94	309.39	1.1	310.18	309.39	0.26

in Table I, as applied to two different crystalline steroids of orthorhombic symmetry. Approximately 1 day was required for each example.

The modified method described here usually results in a gain in both time saved and accuracy. A comparison of these variables for the modified and the conventional film methods is presented in Table II using the orthorhombic crystal tolbutamide for an example. The molecular weight values were calculated using an experimentally determined density value of 1.245.

TABLE II.—COMPARISON OF MODIFIED METHOD AND CONVENTIONAL-FILM METHOD FOR THE ORTHORHOMBIC CRYSTAL TOLBUTAMIDE, MOL. WT. = 270.34

	Conventional Film Method (4)	Modified Method
Unit cell dimensions, Å.	a = 9.07	a = 9.116
	b = 20.14	b = 20.32
	c = 7.78	c = 7.770
Cell volume, Å.^{3}	1421.2	1439.3
Molecular weight, calculated	266.4	269.3
Per cent error in molecular weight	1.45%	0.20%
Time required for result:		
Operator hours	6	4
Total hours	17	8

B. Crystals of Monoclinic Symmetry.—Monoclinic crystals are referred to three unequal axes, two of which, a and c, intersect at an oblique angle, β, and with the b axis normal to the plane of a and c.

The SCO procedure found most useful for the rapid but accurate determination of a monoclinic cell, which again is not necessarily the true unit cell, is as follows.

The crystal is mounted so that the ϕ rotation is about the b crystallographic axis. The procedure is similar to that used for orthorhombic crystals, except that the angular measurement of β (measured in ϕ) is required. Using crystal faces as a guide for a first diffraction signal with χ at 0°, the ϕ and 2θ values are recorded and signals at other ϕ positions sought by scanning both ϕ and 2θ. Only fortuitously would a second signal be found with a ϕ setting 90° from the ϕ setting of the first signal, as would be found with orthorhombic crystals. Several signals at different settings will usually be found, and two ϕ values should be selected for use which are as close to 90° apart as possible. The difference between these ϕ settings is the β angle. The 2θ values at each ϕ setting, when converted to interplanar spacings, may be used to compute the area of a monoclinic cell in the ac plane. Rotation of χ to 90° permits determination of a 2θ value which, when converted, is the cell length along b.

Conventional procedures result in values for a, b, and c, representing true cell edge lengths, and the angle β. The SCO procedure described here measures interplanar spacings which are not necessarily the same as cell edge lengths.

Appropriate interplanar spacings are the same as cell edge lengths in orthorhombic crystals, and one set of interplanar spacings is the same as one cell length in monoclinic crystals (the 90° χ position gives true b). Figure 2 shows a planar section (normal to b) through a monoclinic crystal; a and c are true cell edge lengths; a' and c' are the values obtained from the SCO procedure. The area of the section is $ac \sin \beta$, or $a'c'/\sin \beta$ and the cell volume is $abc \sin \beta$ as conventionally determined, or $a'bc'/\sin \beta$ as determined by the SCO procedure.

Exemplifying the method are the results of the application to the determination of the cell size and the molecular weight of methylprednisolone. This compound crystallizes under normal conditions in the monoclinic system with the true unit-cell constants shown in Table III.

TABLE III.—TRUE UNIT-CELL CONSTANTS FOR THE MONOCLINIC CRYSTAL METHYLPREDNISOLONE

a = 21.78 Å.	Cell volume—1979 Å.^{3}
b = 9.183	n = 4
c = 10.88	Density—1.2544
β = 65° 26.1′	

With the crystal mounted so that the ϕ rotation was about the b axis, and with χ = 0°, signals were found at ϕ settings 65° 26.1′ apart. The 2θ values for these settings gave interplanar spacing values of

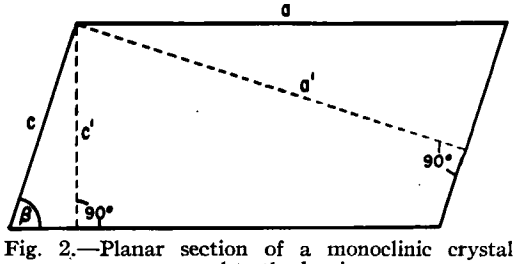

Fig. 2.—Planar section of a monoclinic crystal normal to the b axis.

9.904 Å. and 9.895 Å. Following rotation of χ to 90°, conversion of the measured 2θ value gave a result of 4.591 Å. These values gave a calculated cell volume of 494.69 Å.^{3} which, together with the measured density value of 1.2544, allowed the calculation of the molecular weight, 373.8. (True molecular weight, 374.46.)

The volume of the subcell measured by the SCO procedure is one-fourth that of the true unit cell, as two of the measurements were of hemiaxes. Again, the fact that the modified method measures a fractional cell is of no consequence when used for molecular weight determinations. In fact, the method quite often gives the molecular weight directly, as in the example just cited, rather than some multiple of it.

As with the determination of orthorhombic cell volumes, a gain in accuracy can sometimes be achieved with monoclinic cells by combining SCO data and at least partial powder-diffraction data. The probability of doing so at a saving of time is not so great as with orthorhombic crystals, however, due to the greater complexity of the indexing problem. This is so when the indexing is done manually. With the aid of machine methods, however, the gain in accuracy can be made with no time loss. By programming a digital computer[3] to solve the following equation[4] for all values of h, k, and l,

$$d = \frac{1}{\sqrt{\frac{\frac{h^2}{a^2} + \frac{l^2}{c^2} - \frac{2hl}{ac}\cos\beta}{\sin^2\beta} + \frac{k^2}{b^2}}}$$

given a, b, c, and β, a list of d spacings can be produced. This list is essentially a synthetic powder pattern whose d values are of no greater accuracy than the SCO data but which is accurately indexed. Comparison with the experimental powder pattern (accurate d values, but not indexed) results in accurate indexing of the accurate d values, which may then be used to compute the cell volume.

C. Crystals of Triclinic Symmetry.—Triclinic crystals are referred to three unequal axes, all intersecting at oblique angles. Such crystals have undergone such an extreme decrease in symmetry when compared to monoclinic crystals that the cell volume calculation is highly involved. Further, measurements of three oblique angles give rise to major sources or error. When using conventional film techniques, one almost invariably gains in both time and accuracy of the cell-volume determination when time is invested in converting the organic crystal to one of higher symmetry through either a polymorphic change, solvation (the degree to be later determined), or derivativization.

Although the SCO is ideally suited for the determination of the true unit cell edge lengths and true interaxial angles, for purposes of molecular weight determinations the true cell constants need not be found. As pointed out by Bunn as early as 1945 (4), the volume of a triclinic cell is one axis length multiplied by the area of that axis projection. Using film techniques, one could measure the layer-line spacings, achieving the axis length, and theoretically, from equatorial spacings, the dimensions of the axis projection. Finding the axis projection required the determination of an oblique angle, however, as well as several reflection orders of two different dimensions, all from one row of superimposed spots. With film techniques, then, the axis projection method is still quite complicated.

The availability of the SCO allows easy application of the axis-projection method to triclinic cells. Errors are significantly reduced by the reduction in the number of required angles measured from three to one and even when the SCO method is combined with a partial film method (to measure one axis length) there is a great saving of time. As mentioned above, the SCO is capable of measuring all triclinic cell constants. Experience in these laboratories has shown, however, that the most rapid procedure with acceptable accuracy involves a combination of SCO and film techniques. This procedure is described below.

With the crystal mounted in the SCO ($\chi = 0°$) so that one crystal axis is coincident with ϕ, scans of ϕ and 2θ are made until signals corresponding to two different settings of ϕ are found. The 2θ values are converted to interplanar spacings and recorded along with the angle between the two ϕ settings. The product of these spacings, divided by the sine of this angle, is the area of the axis projection. The goniometer head, together with the undisturbed mounted crystal, can then be transferred to a film camera for a standard rotation photograph. The layer-line spacings of the rotation photograph allow the calculation by standard techniques of the axis length. The product of this length and the area of the axis projection is the volume of the cell. The cell measured in this manner is not necessarily the true unit cell but bears some whole number relation to it so that an accurate molecular weight value can be found.

In order to compare the conventional and the modified techniques, as applied to a triclinic crystal, cholesterol may be used as an example. The true unit cell constants of cholesterol (5) are shown in Table IV. The volume of a triclinic cell is $v = abc$

TABLE IV.—TRUE UNIT-CELL CONSTANTS FOR THE TRICLINIC CRYSTAL CHOLESTEROL

$a = 14.10$	$\alpha = 94.60°$
$b = 33.75$	$\beta = 90.00°$
$c = 10.46$	$\gamma = 95.72°$

$\sin\beta \sin\gamma \sin\delta$ where

$$\sin\frac{\delta}{2} = \sqrt{\sin\frac{(\alpha - \beta + \gamma)}{2}\sin\frac{(\alpha + \beta - \gamma)}{2}}$$

The calculated volume of the cholesterol unit cell is 4939.7 Å.^{3}. All six constants listed above were required to calculate this value.

Applying the modified method with the crystal mounted with c and ϕ coincident, values of 33.60 Å. and 13.97 Å. are recorded from two separate ϕ settings, which are 95°42′ apart. These correspond to the 010 and 100 reflections. After determining

[3] The author is grateful to Dr. O. S. Carpenter of The Upjohn Co. for programming and operation supervising of the Burroughs E-102 digital computer.

[4] It may be noted that for orthorhombic crystals, where $\beta = 90°$, this equation reduces to that presented in the previous section, footnote 1. The same computer program may therefore be used to index powder patterns of both orthorhombic and monoclinic symmetry.

the c axis length, 10.46 Å., from a single rotation photograph, a simple calculation gives v'

$$v' = \frac{33.60 \text{ Å.} \times 13.97 \text{ Å.} \times 10.46 \text{ Å.}}{\sin 95°42'} = 4934.2 \text{ Å.}^3$$

This value agrees well with the previously calculated v for the true unit cell, 4939.7 Å.^{3}. It should be noted that v' was obtained by a simpler procedure which is usually more rapid and, as noted above, the requirement of measuring only one, instead of three angles, usually results in a significant gain in accuracy.

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Effect of Drugs on Survival Time from Scorpion Envenomation

By LEO GREENBERG and JAMES W. INGALLS

Androctonus australis **venom at various dilutions was administered to matched groups of mice and the effects on survival time and number determined for various classes of therapeutic agents. Meperidine and other narcotics were consistently deleterious to envenomed animals as were alcohol and calcium acetylsalicylate carbamide. Neither corticosteroids nor muscle relaxants were beneficial. With tranquilizing agents, meprobamate tended to increase survival time, while reserpine markedly curtailed survival.**

In previous papers from our laboratories, we have investigated the interaction of tranquilizing drugs and various toxic agents in laboratory animals. These challenges have included curare (1), bacterial exotoxins (2–4), bacterial endotoxins (3, 4) and septicemias (2, 3). Presently, reports are in preparation on amoebiasis and trichinosis. It was of interest therefore to determine if the profound alterations in survival time evinced with tranquilizer treatment in the previous conditions could be demonstrated with arthropod venom. Moreover, it was felt that such a study might have more than academic interest since scorpion envenomation represents a major hazard to man and animals throughout much of the tropical and semitropical areas of the earth's surface. In some areas, principally North Africa, the danger from scorpions is greater than from venomous snakes (5).

It is known that the pharmacological actions of scorpion venom from a great variety of genera and species are quite similar (6) and that species widely separated by geography contain venom components in common (7). Symptoms of envenomation include muscular contractions, salivation, respiratory paralysis, and vasoconstriction. It is suggested that these indicate the existence of a substance or substances with strong parasympathetic activity acting in a similar manner to serotonin (8).

In the present study, in addition to tranquilizing drugs, other agents of real or imagined therapeutic significance in the envenomed state were included for comparison. The narcotics meperidine,[1] cocaine, codeine, and morphine were tested in light of the findings of Stahnke (9, 10) that both morphine and meperidine are undesirable therapeutic agents for use in envenomation from the Arizona scorpion, *Centruroides sculpturatus*. The analgesics calcium acetylsalicylate carbamide[2] and dextropropoxyphene hydrochloride[3] were included as possible substitutes for the narcotic agents in controlling pain. Methocarbamol[4] was tested on the basis of its reported value in cases of black widow spider bites (11), and two other muscle relaxants, carisoprodol[5] and phenyramidol hydrochloride,[6] were included for comparison. In view of the reported parasympathomimetic action of scorpion venoms, the antiparasympathetic scopolamine was included in this study. Alcohol was used in keeping with the long standing proscription against whisky in snake bite, and the barbi-

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turates phenobarbital and hexobarbital were tested as possible substitutes for the narcotics as sedating agents. Finally, the corticosteroids were included in keeping with their role in the stress state, and in light of recent reports on the relation of cortisone and the effects of Egyptian scorpion venom (12) and protection against cobra venom by hydrocortisone (13).

MATERIALS AND METHODS

The animals used in this study were 20–25-Gm. CF1 male mice (Carworth Farms), caged in small groups and kept in the thermostatically controlled animal house for several days prior to use. In all experiments food was withheld after the venom challenge for at least 12 hours but water was allowed *ad libitum*.

Lyophilized venom of the North African scorpion, *Androctonus australis*[7] was diluted with sterile, isotonic saline immediately prior to each experimental series. Preliminary evaluation of this venom indicated an intraperitoneal LD_{50} of approximately 0.05 mg. for mice, which compares favorably with the published intramuscular LD_{50} of 0.091 mg. (5). In all experiments, 0.2 ml. of diluted venom was injected intraperitoneally.

All drugs were purchased locally or supplied through the courtesy of their manufacturers, diluted with sterile, isotonic saline, and injected intraperitoneally in a volume of 0.2 ml. approximately 30 minutes prior to venom challenge. Insoluble substances, such as meprobamate, were first suspended in a small volume of 5% acacia solution. Preliminary experiments established that at the doses employed none of the drugs were markedly deleterious to mice of the weight and strain used in these tests. In all experiments, matched groups of ten or more animals were used; experimental and control animals in any given series were inoculated within a few minutes of one another. Control animals all received 0.2 ml. of sterile saline solution. Death times were recorded at the cessation of respiration and heart beat.

RESULTS AND DISCUSSION

Following the establishment of an approximate LD_{50} for the venom sample at 0.2 ml. of 1:20,000 dilution, fifteen therapeutic agents were tested for their ability to alter survival time and number, the results being plotted in Fig. 1. From these data, several distinct patterns of response are evident. One group of agents, including the barbiturates, corticosteroids, the antiparasympathetic scopolamine, and the muscle relaxant methocarbamol, showed no evident difference from the control animals at the dose employed. Five of the drugs used, carisoprodol, ethanol, calcium acetylsalicylate carbamide, phenyramidol hydrochloride, and dextropropoxyphene hydrochloride markedly shortened survival time compared to control animals. Still more marked, both in shortening of survival time and in increase in mortality, were chlorpromazine, meperidine, and reserpine. On the other hand, of all of the agents tested, only meprobamate evidenced a moderate increase in survival time over control animals.

A similar group of drugs, in some cases at different dose levels, was then evaluated using an overwhelmingly lethal dilution of venom. These data are summarized in Table I. A general agreement with the previous figure is evident. Of all the agents tested, reserpine most sharply reduced survival time while increasing doses of meprobamate significantly increased survival time in the envenomed animals. At a dose of 50 mg./Kg., meperidine again shortened survival time as did calcium acetylsalicylate carbamide and ethanol while corticosteroid therapy again showed no significant alteration in survival time.

On the basis of these data, further studies were conducted with meprobamate and reserpine. With meprobamate treatment at various dose levels, time of administration and venom dilutions, a general trend toward increased survival was noted. However, this was neither of such magnitude nor consistency as to merit serious clinical consideration. With reserpine, however, a marked and consistent pattern of decreased survival time was demonstrated. Table II indicates the effect of a single dose of reserpine on the survival time and number in matched groups of animals inoculated with eight dilutions of scorpion venom. At all dilutions, mean survival time was decreased with reserpine treatment, and at or near the LD_{50}, mortality was increased.

In speculating on the mode of action by which reserpine exerts its deleterious effect in these experiments, it is tempting to implicate serotonin. Mention has already been made of the similarity in action between scorpion venom and serotonin (8).

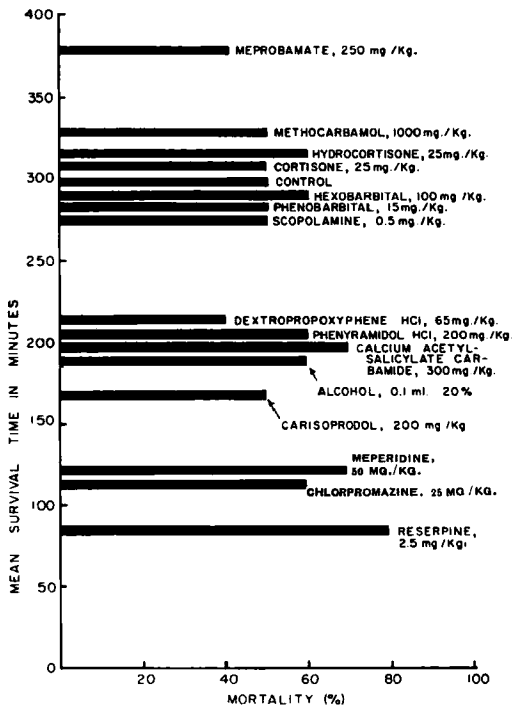

Fig. 1.—Effect of various drugs on matched groups of mice inoculated with 0.2 ml. of 1:20,000 venom (LD_{50}).

[7] Supplied through the courtesy of Dr. Lucien Balozet, Institut Pasteur d'Algérie, Algiers, French North Africa.

TABLE I.—EFFECT OF DRUGS ON SURVIVAL TIME OF MATCHED GROUPS OF MICE INOCULATED WITH A LETHAL DOSE OF SCORPION VENOM (0.2 ML., 1:2000 DILUTION)

Drug	Dose/Kg.	Survival Time, min. ± S.E.	Significance
Reserpine	2.5 mg.	14.5 ± 0.96	P = <0.01
Alcohol, 20%	0.1 ml.	14.9 ± 0.23	P = <0.001
Calcium acetylsalicylate carbamide	300 mg.	15.1 ± 1.0	P = <0.01
Hydroxyzine	25 mg.	15.2 ± 0.76	P = <0.01
Meperidine	50 mg.	15.2 ± 1.08	P = <0.02
Codeine	120 mg.	17.2 ± 0.03	P = <0.05
Cocaine	50 mg.	17.2 ± 3.0	P = >0.6
Codeine	60 mg.	17.2 ± 1.48	P = >0.3
Morphine	30 mg.	17.5 ± 1.11	P = >0.3
Meperidine	25 mg.	18.6 ± 1.12	None
Hydrocortisone	25 mg.	18.7 ± 0.76	None
Control	...	20.6 ± 1.01	...
Chlorpromazine	25 mg.	20.8 ± 0.95	None
Cortisone	25 mg.	20.8 ± 4.10	None
Morphine	15 mg.	22.5 ± 2.27	P = >0.3
Meprobamate	200 mg.	24.1 ± 1.76	P = <0.01
Meprobamate	250 mg.	26.0 ± 0.58	P = <0.001
Meprobamate	300 mg.	28.1 ± 1.08	P = <0.001

TABLE II.—EFFECT OF INCREASING DILUTION OF VENOM ON SURVIVAL TIME OF MATCHED GROUPS OF MICE WITH AND WITHOUT RESERPINE TREATMENT (2.5 mg./Kg., i.p.)

	Dilution	Mortality, %	Mean Survival Time, min.
Control	1:4,000	100	29.5
Reserpine		100	15.0
Control	1:6,000	100	34.0
Reserpine		100	24.0
Control	1:8,000	100	39.4
Reserpine		100	28.5
Control	1:12,000	100	67.5
Reserpine		100	33.0
Control	1:16,000	100	226
Reserpine		100	163
Control	1:20,000	50	425[a]
Reserpine		100	300
Control	1:40,000	0	...
Reserpine		20	...
Control	1:80,000	0	...
Reserpine		0	...

[a] Mean survival time of those animals which died.

More recently, it has been reported that serotonin is present in the venom of all species of scorpions studied, and that this ubiquitous finding cannot be fortuitous (14). Since it is well known that reserpine administration results in the release of considerable serotonin into the circulation, it is indeed possible that the overwhelming combination of exogenous serotonin in scorpion venom and endogenous serotonin from reserpine injection sharply curtails the survival time of such animals.

SUMMARY AND CONCLUSIONS

1. Venom of the North African scorpion, *Androctonus australis* was administered intraperitoneally to mice in dilutions ranging from the LD_{50} to the overwhelmingly lethal. Numerous drugs of possible significance in scorpion envenomation were tested to determine their effect on survival time.

2. Of the drugs tested, only meprobamate evidenced a trend toward increased survival in envenomed animals although this was not of sufficient magnitude to merit clinical trial.

3. Our results support previous studies demonstrating the profoundly deleterious effects of meperidine in scorpion envenomation and indicate a similar response, although not as marked, with other narcotic drugs.

4. Ethanol and calcium acetylsalicylate carbamide consistently reduced survival time while corticosteroid and barbiturate treatment showed little effect. Antiparasympathetic and muscle relaxant drugs proved of little significance or were deleterious.

5. Reserpine treatment consistently and markedly reduced survival time at all venom dilutions tested, and the likelihood that this effect is related to serotonin release has been discussed.

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Dissolution Rates of Finely Divided Drug Powders II

Micronized Methylprednisolone

By W. I. HIGUCHI†, E. L. ROWE, and E. N. HIESTAND

The dissolution rate of micronized methylprednisolone in aqueous solutions was measured. Also, the particle size distribution was measured and an approximate distribution function was found. This function was incorporated into the theory for the diffusion-controlled dissolution of finely divided heterodisperse powders. The reasonably good agreement between experiment and theory suggests that the rate-determining step, in this instance, is diffusion of the drug in the aqueous phase. The possible effects of agitation, sedimentation, particle shapes, and the variation of solubility with particle size have been considered. The composite effects of these may account for the deviations between experiment and theory.

RECENTLY (1), the theoretical aspects of the liquid phase diffusion-controlled release characteristics of finely divided drug powders were examined in detail. The effects of particle size, size distribution, and drug solubility were considered in the formulation of a procedure for predicting the release *versus* time curves for powders in the region of particle size $\lesssim 25\,\mu$.

In this report, experimental studies of the dissolution rates of micronized methylprednisolone[1] in water are presented. The agreement of the data with theory is very satisfactory and demonstrates the usefulness of the theoretical procedure.

EXPERIMENTAL

Dissolution Rate Studies.—About 2.20 mg. of micronized methylprednisolone was added at zero time to 100 ml. of water in bottles which were rotated at 6 r.p.m. in a constant temperature bath maintained at 25°. The bottles were held in fixed positions on rotating wheels. Thus, mild agitation by tumbling action was achieved. Pure water was used in one set of experiments. In the other runs, the water was partially saturated with methylprednisolone to the extent of 30, 50, 70, and 90% of saturation so that a more rigorous test of the theory would be possible. At predetermined times bottles were removed and filtered rapidly, and then the filtrates were assayed spectrophotometrically.

Particle Size Analysis.—The Coulter counter[2] with the 100-μ aperture was used to determine the distribution of sizes. A 1% sodium chloride solution saturated with methylprednisolone was employed as the vehicle.

THEORETICAL CALCULATIONS

As before (1), we may write

$$a = \left(a_o^2 - \frac{2D\Delta Ct}{\rho}\right)^{1/2} \qquad \text{(Eq. 1)}$$

where a_o is the initial particle radius, a is the particle radius at time t, D is the diffusion coefficient of the drug molecule in water, ρ is the solid drug density, and $\Delta C = C_s - C_o$ where C_s is the solubility of the drug and C_o is the drug concentration in solution. While Eq. 1 applies strictly to the case where ΔC remains constant, it is easy to correct for the effects of the variation in ΔC, as will be shown later.

As most milled materials follow the log-normal distribution of sizes, Eq. 1 may be combined with a suitable function which approximates the log-normal function to give the expression for the dissolution rate of powders. This was done in the previous paper (1) and represents a generalization of the present treatment. Since in this study the initial size distribution of the methylprednisolone powder was determined with the Coulter counter, the present analysis utilizes the measured particle size distribution and constitutes a test of the applicability of the theory.

Equation 1 describes the dissolution of a single particle of initial size a_o. Consider now the situation in which there is initially a *distribution* of sizes. Let

$$n = n(a_o) \qquad \text{(Eq. 2)}$$

represent the measured distribution function. The quantity n is the initial ($t = 0$) number of particles between the sizes a_o and $a_o + da_o$. The total number of particles in the system at $t = 0$ is then

$$N = \int_{a_{so}}^{a_{lo}} n(a_o)da_o \qquad \text{(Eq. 3)}$$

where a_{lo} and a_{so} are the radii of the smallest and the largest particles, respectively, at $t = 0$.

The total mass of undissolved drug at $t = 0$ is

$$M_o = \int_{a_{so}}^{a_{lo}} \frac{4}{3}\pi a_o^3 \rho n(a_o)da_o \qquad \text{(Eq. 4)}$$

Now at any $t > \frac{a_{so}^2\rho}{2D\Delta C}$, the total amount of undissolved drug will be

$$M = \int_{a_{ot}}^{a_{lo}} \frac{4}{3}\pi\rho a^3 n(a_o)da_o \qquad \text{(Eq. 5)}$$

Here a is given by Eq. 1 and the lower limit of the integral, a_{ot}, is given by

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[1] Medrol, from The Upjohn Co.
[2] Marketed by Coulter Industrial Sales, Elmhurst, Ill.

$$a_{ot} = \left(\frac{2Dt\Delta C}{\rho}\right)^{1/2} \quad \text{(Eq. 6)}$$

This limit a_{ot} is the initial ($t = 0$) size of the particle which dissolves completely at time t.

Inserting the expression for a from Eq. 1 into Eq. 5 gives

$$M = \int_{a_{ot}}^{a_{lo}} \frac{4}{3}\pi\rho\left(a_o^2 - \frac{2Dt\Delta C}{\rho}\right)^{3/2} n(a_o)da_o \quad \text{(Eq. 7)}$$

So the fraction, Q, of drug undissolved at time t is

$$Q = \frac{M}{M_o} = \frac{\int_{a_{ot}}^{a_{lo}} \left(a_o^2 - \frac{2Dt\Delta C}{\rho}\right)^{3/2} n(a_o)da_o}{\int_{a_{so}}^{a_{lo}} a_o^3 n(a_o)da_o} \quad \text{(Eq. 8)}$$

and hence the per cent release, R, is given by

$$R = 100\,(1 - Q) \quad \text{(Eq. 9)}$$

APPLICATION

Release Rate Data on Micronized Methylprednisolone.—In Fig. 1 we have the Coulter counter data for micronized methylprednisolone for which the dissolution rate studies were carried out. In the figure the smooth curve corresponds to the following empirical equation for the distribution function

$$n(a_o) = \frac{K}{a_o^2} \quad \text{(Eq. 10)}$$

where K is a constant. For a_{lo} and a_{so} the values of 0.5 μ and 9.0 μ were chosen respectively to give the good fit shown in Fig. 1. These limits correspond to a size range of 1–18 μ diameter and agree with microscopic sizing of micronized methylprednisolone.

We may now substitute Eq. 10 into Eq. 8 to get

$$Q = \frac{\int_{a_{ot}}^{a_{lo}} \frac{1}{a_o^2}\left(a_o^2 - \frac{2Dt\Delta C}{\rho}\right)^{3/2} da_o}{\int_{a_{so}}^{a_{lo}} a_o da_n} \quad \text{(Eq. 11)}$$

Integrating over the limits for both integrals in Eq. 11 and substituting for a_{ot} from Eq. 6, we obtain

$$Q = \frac{2}{(a_{lo}^2 - a_{so}^2)}\left[\left(\frac{a_{lo}}{2} + \frac{2Dt\Delta C}{\rho a_{lo}}\right)\left(a_{lo}^2 - \frac{2Dt\Delta C}{\rho}\right)^{1/2} + \frac{3Dt\Delta C}{\rho} ln\left\{\frac{\left(\frac{2Dt\Delta C}{\rho}\right)^{1/2}}{a_{lo} + \left(a_{lo}^2 - \frac{2Dt\Delta C}{\rho}\right)^{1/2}}\right\}\right] \quad \text{(Eq. 12)}$$

Now we have the following information on methylprednisolone: ρ = density = 1.28 Gm./ml.;[3] MW = molecular weight = 374; C_s = solubility = 7.4×10^{-5} Gm./ml. at 25°. We may calculate the diffusion coefficient, D, by means of the Stokes-Einstein relation

$$D = \frac{kT}{6\pi vs} \quad \text{(Eq. 13)}$$

where k = Boltzmann's constant = 1.37×10^{-16}; T = 300° Kelvin; v = viscosity $\simeq$ 0.01 poise for water; s = molecular diffusion radius for methylprednisolone, calculated from density and molecular weight = 4.7×10^{-8} cm. ±10%. Equation 13 then gives $D = 4.7 \times 10^{-6}$ cm.^{2} sec.$^{-1}$.

Inserting all of these values into Eq. 12 and combining with Eq. 9 gives the per cent released, R,

$$R = 100\left[1 - (1.12 \times 10^3 + 2.02 \times 10^4\, t\Delta C)(8.1 \times 10^{-7} - 7.34 \times 10^{-6}\, t\Delta C)^{1/2} - 62t\Delta C \log_{10}\left\{\frac{2.71 \times 10^{-3}\,(t\Delta C)^{1/2}}{9 \times 10^{-4} + (8.1 \times 10^{-7} - 7.34 \times 10^{-6}\, t\Delta C)^{1/2}}\right\}\right] \quad \text{(Eq. 14)}$$

Now in the derivation of Eq. 1 it was necessary to assume that ΔC was constant with time. Hence, Eq. 14 strictly applies only to the case of large undersaturation, *i.e.*, for $\Delta C \simeq C_s \gg C_o$. In order to apply Eq. 14 to the present release data (for which ΔC decreases with time), an approximation procedure was employed. The procedure may be carried out in the following manner: First, make a plot of R *vs.* $t\Delta C$ by means of Eq. 14. Then select R values from 0 to 100 so that ΔC does not change too greatly from one R value to the next (*i.e.*, changes in ΔC from one R value to the next must be small compared to ΔC itself). From the release rate experiments we know $\Delta C = C_s - C_o$ for any R value. Hence $\Delta t = t_{n+1} - t_n$ corresponding to R_{n+1} and R_n may be determined. Then finally, the sum of Δt values may be plotted against R to give the R *vs.* time curves.

Figure 2 shows the theoretical curves obtained by means of Eq. 14 and the approximation procedure just outlined. The experimental data are represented by the points in the figure. Because no empirical factor was used in the theoretical calculations, the agreement between the experiments and theory must be regarded as highly satisfactory

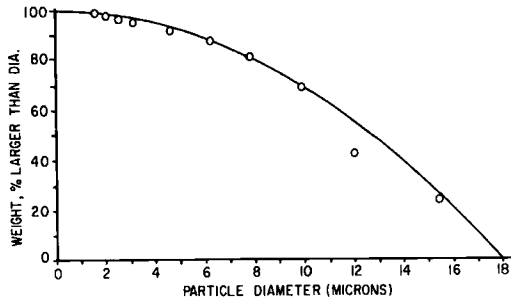

Fig. 1.—Distribution of particle sizes for micronized methylprednisolone obtained with the Coulter counter. Smooth curve is in accordance with $n = \frac{K}{a_0^2}$.

[3] Courtesy of Dr. J. W. Shell.

despite some deviations. Clearly, the most likely rate-determining step is the rate of diffusion of methylprednisolone in water. It is possible that other processes such as surface nucleation or surface orientation might contribute to the dissolution rate, but certainly, even if they do, they are not very important for micronized methylprednisolone.

It is apparent that the experimental release rates are always somewhat greater than theory at small values, and always somewhat less at large t. Several possible reasons may be put forward to explain these deviations.

Firstly, it is worthwhile to mention that if a particle size-distribution function corresponding to a somewhat broader distribution were employed in the theoretical calculations, an almost perfect agreement would be obtained for cases A through D. It is possible that the distribution curve (Fig. 1) determined with the Coulter counter does not exactly represent the true initial size distribution, particularly for the smaller sizes. This might be attributed to the tendency for small particles to be more soluble than the larger ones. Thus, if the vehicle were saturated with a sample of methylprednisolone, it might have been undersaturated with respect to the small particles of a subsequently added sample. In case E (see Fig. 2), for which the dissolution medium was initially 90% saturated, the final concentration of methylprednisolone exceeded the solubility (7.40 mg. per 100 ml.) of prednisolone by about 3 or 4%.[4] This might also be explained by the greater solubility of the smaller particles. It was furthermore noted that in the determination of the methylprednisolone solubility, which was found by adding a three-fold excess of methylprednisolone to water and following the methylprednisolone concentration as a function of time, a maximum value several per cent greater than the final constant value was observed. This is consistent with the behavior in case E and with the possibility that the Coulter counter did not give correct weighting to the small particles.

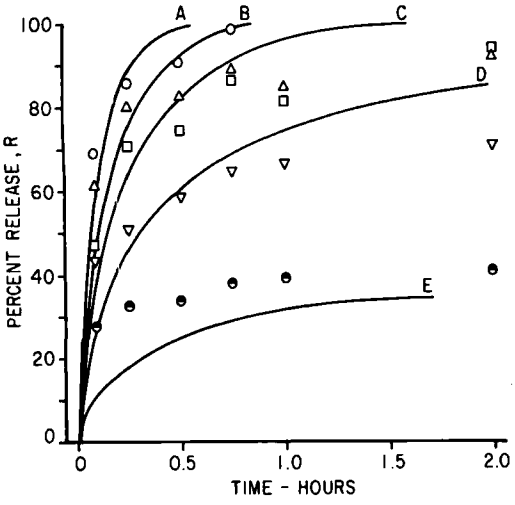

Fig. 2.—Experimental and theoretical release of drug from micronized methylprednisolone. Theory A, pure water, experiment ○; theory B, 30% presaturation, experiment △; theory C, 50% presaturation, experiment □; theory D, 70% presaturation, experiment ▽; theory E, 90% presaturation, experiment ●.

The theory assumes that the particles are spheres. The influence of shape variations may be estimated from the electrostatic theory (2) of conducting ellipsoids. Ellipsoids with axial ratios of two or so will dissolve at about a 5% greater rate than spheres of the same volume. Since the micronized methylprednisolone particles were found to be relatively isometric under the microscope, the shape effects were probably unimportant.

Occasionally, clumping of some of the particles was observed during the dissolution experiments. Some of the scatter as well as the low rates at large t values may be explained on this basis. In general, however, the methylprednisolone samples dispersed readily.

Another factor contributing to the deviations is the Stokes law calculated value for the diffusion coefficient. The uncertainty here is estimated to be around 10–20%. If a smaller value for D were used, the theoretical curves in Fig. 2 would be expanded in proportion to t along the t-axis, or if a larger D value were employed, the curves would be linearly contracted.

Finally, the effects of stirring and the sedimentation of particles will affect the dissolution rate to a certain extent. Recent studies by Nielsen (3) show that for methylprednisolone particles in the size range $\lesssim 25\ \mu$, the hydrodynamic effects arising from sedimentation should be small, the order of a few per cent, insofar as the dissolution rate is concerned. Furthermore, the effects due to moderate stirring should have been also small for these particle sizes since the local accelerations and decelerations in the medium probably were at most only comparable to that due to gravity, and since velocity gradients were probably $\lesssim 1.0\ \text{sec.}^{-1}$.

Thus, the composite effect of these uncertainties may very well account for the deviations. Future experiments will dwell upon these factors more thoroughly.

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Permeability of Excised Human Keratin to Lipid-Soluble Substances II

By ROBERT E. DEMPSKI†, DALE E. WURSTER, and STANLEY L. BECKER

Data are presented which compare the rate of penetration through keratin tissue of 2-hydroxystearic acid and three mixtures composed mainly of lipid-soluble esters, with oleyl alcohol. The hydroxy acid which was strongly adsorbed by keratin had a very slow rate of penetration. The ester mixture with the lowest acid number showed rapid penetration. The mathematical interpretation of the data indicated that the transport occurred by a diffusional process.

A PREVIOUS study has shown that certain lipid-soluble substances were able to penetrate sections of excised human callous tissue (1). It was also observed that substances such as linolenic and linoleic acids which were adsorbed by human keratin from *n*-heptane solutions manifested relatively slow permeability rates in sections of this tissue. This paper is a continuation of that study, and contains permeability data on a hydroxy fatty acid and three different lipid-soluble mixtures containing esters as the primary component.

Since it can be shown that the distribution coefficient (2) can affect the rate of penetration, the influence of this factor was also investigated.

EXPERIMENTAL

Procedure.—The apparatus, including the diffusion cells and the method employed to follow the permeability rate of the various lipid-soluble substances in human keratin tissue, were essentially the same as described in a previous paper (1). The preparation of the keratin tissue sections and the assay for oleyl alcohol were also the same.

The 2-hydroxystearic acid concentration in each side of the diffusion cell was determined by titrating with a standard solution of 0.001 *N* sodium hydroxide in *n*-butanol with a microburet under an atmosphere of nitrogen using *p*-naphtholbenzein as the indicator. The initial concentration of the acid was 1.0×10^{-6} moles per ml. Use of this low concentration was necessitated due to the low solubility of the acid in *n*-heptane. The reference penetrant, oleyl alcohol, was used in the same concentration in this case.

The lipid-soluble mixtures (liquid lanolin Nos. 1, 2, and 3), consisting mainly of esters (3, 4), were assayed as previously described (1) by the hydroxylamine test using 2 ml. of the *n*-heptane solution and 3 ml. of ether. The concentration of the mixtures and the oleyl alcohol in this case was based on mg./ml. (from 5.26 to 6.29 mg./ml.) rather than on a molar basis. This, of course, was necessary since these penetrants were composed mainly of a mixture of esters rather than a pure component. Naturally, minor concentrations of other components such as acids and alcohols were also present in the mixtures.

Distribution coefficients were determined according to the following method: Five grams of powdered (200 mesh) ether-extracted keratin tissue was placed in a glass-stoppered vessel; a measured volume (100 ml.) of a *n*-heptane solution of the lipid-soluble test substance was then added, and the mixture was continuously agitated and allowed to reach equilibrium at 25°. Samples of the clear *n*-heptane solution were subsequently removed and assayed according to the described procedure for each agent.

Acid numbers were determined for each lipid-soluble mixture according to the U.S.P. XVI procedure.

RESULTS AND DISCUSSION

The results of the permeability studies again showed a wide variation in the permeability coefficients for the callous tissue from different individuals to a specific penetrant. Therefore, as previously explained, each penetrant and oleyl alcohol was tested against alternate layers from the same piece of tissue (1). The permeability coefficients, K, are average values and were calculated according to the equation (5),

$$\log (C_o - 2C_b) = (-2K/2.303)t + \log C_o \quad \text{(Eq. 1)}$$

where C_o is the initial concentration of solution to which the keratin barrier was exposed and C_b is the concentration at the time, t, on the other side of the barrier in the diffusion cell.

TABLE I.—RATIO OF THE PERMEABILITY COEFFICIENTS OF VARIOUS TEST PENETRANTS TO THAT OF THE REFERENCE MATERIAL, OLEYL ALCOHOL

Test Penetrant	$\frac{K \text{ (test)}}{K \text{ (oleyl)}}$
Cholesteryl acetate	0.942
Cholesteryl oleate	0.298
Oleic acid	0.476
Linolenic acid	0.157
Linoleic acid	0.039
2-Hydroxystearic acid	0.028
Liquid lanolin No. 1	1.235
Liquid lanolin No. 2	2.029
Liquid lanolin No. 3	0.767

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Since biological variation makes it impossible to directly compare permeability constants of various penetrants tested on different tissue sections, oleyl alcohol was again used as a reference. By calculating the ratio of the permeability coefficient for the test penetrant to that obtained for oleyl alcohol on the alternate tissue layers, the values obtained for each agent were reduced to a common basis. In Table I the ratios for various agents investigated previously (1) and those included in this study are shown.

Obviously, those substances in the table having the larger values have greater permeability coefficients as a result of their more rapid penetration rates in keratin tissue. It can also be observed, as previously reported (1), that some substances which are strongly adsorbed by keratin have low penetration rates. Thus, 2-hydroxystearic acid was the most strongly adsorbed of all the substances tested (6) and had the lowest permeability rate.

In the case of the various liquid lanolin preparations tested, these, too, appear to follow the same pattern. The liquid lanolin No. 1 sample had an acid number of 1.69. The No. 2 liquid lanolin sample was characterized by its low hydroxyl content and an acid number of 1.17, whereas the No. 3 liquid lanolin had an acid number of 6.79. Since wool fat contains both unsaturated and 2-hydroxy acids (7), it is reasonable to expect that the No. 2 liquid lanolin would have the greatest, and No. 3 the smallest, permeability coefficient of these three mixtures.

Although the relative position of each of the three mixtures is no doubt correct, an additional theoretical point should be mentioned. It was necessary, for reasons already given, to follow the change in concentration of the lanolin mixtures on a weight per unit volume basis and thus, the Beer's plot which was employed to follow the change in concentration with time was constructed on a similar basis. However, the material diffusing through the keratin membrane could differ from the composition of the mixture. Since the intensity of the color produced in the assay is directly proportional to the ester concentration, the results could be misleading. For example, if the material diffusing through the membrane contained a higher concentration of esters or a larger fraction of the lower molecular weight esters than an equivalent weight of the entire mixture, the absorbance would be greater. This would then show an apparent fast rate of penetration for the mixture.

In the derivation of the previous equation, the permeability coefficient is shown to be equal to several constants

$$K = \frac{AD\,(DC)}{VL} \qquad \text{(Eq. 2)}$$

where K is the permeability coefficient, A is the cross-sectional area, D is the diffusion coefficient, DC is the distribution coefficient, V is the volume, and L is the length of the diffusional pathway. Thus, if the quantity, AD/VL is constant, the permeability coefficient should vary directly with the distribution coefficient. In Table II, the apparent distribution coefficients between powdered keratin and n-heptane for several lipid-soluble substances are shown.

In the case where the penetrating molecule is soluble in a liquid or semisolid medium in contact with a similar medium it can be shown that the permeability coefficient is directly proportional to the distribution coefficient (2). However, in the case where a liquid is in contact with a solid and the solute is strongly adsorbed by the solid, such as in the case of the powdered keratin-n-heptane systems employed in this study, the above relationship was not found to exist.

Microscopic examination of the sections of callous tissue showed them to be free of orifices through which the penetrant could stream (8). Also, the two sides of the permeability cell did not reach equilibrium even after being agitated for 10 days. Thus, the experiment and the mathematical interpretation of the data indicated that a true diffusional process occurred. This lends support to the theory that transepidermal penetration, at least through the stratum corneum, can occur.

TABLE II.—DISTRIBUTION COEFFICIENTS OF VARIOUS AGENTS BETWEEN POWDERED EXCISED HUMAN KERATIN AND NORMAL HEPTANE

Test Agent	Distribution Coefficient
Oleyl alcohol	0.40
Cholesteryl acetate	0.63
Cholesteryl oleate	0.64
Oleic acid	1.72
Linolenic acid	1.19
Linoleic acid	0.58
2-Hydroxystearic acid	36.00
Liquid lanolin No. 1	0.53

SUMMARY

The penetration rates in excised human callous tissue of 2-hydroxystearic acid and three liquid lanolin preparations were determined.

The 2-hydroxystearic acid which was strongly adsorbed by human keratin showed the lowest permeability coefficient of all the substances tested.

The acid numbers of the three liquid lanolin preparations were also determined. The liquid lanolin characterized by the lowest acid number and low hydroxyl content showed the most rapid penetration rate. The preparation having the highest acid number showed the slowest penetration rate of the three mixtures.

The apparent distribution coefficients for several lipid-soluble substances in a powdered keratin-n-heptane system were determined.

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Spectrophotometric Determination of Chloramphenicol and Tetracycline Hydrochloride in Mixtures

By R. C. SHAH, P. V. RAMAN, and B. M. SHAH

A rapid method for the spectrophotometric determination of chloramphenicol and tetracycline hydrochloride in binary mixtures without prior separation is described. Chloramphenicol is determined by the measurement of difference in (extinction) values (ΔE) at 282 mμ and 233 mμ, and tetracycline hydrochloride is determined by measurement of absorbance at 380 mμ, using 0.1 *N* ammonium hydroxide as the solvent.

With the extensive use of antibiotics, and the increased prevalence of mixed antibiotic therapy, the pharmaceutical analyst is faced with the problem of developing quick and reliable methods for the assay of individual components in mixtures. Spectrophotometric and other physicochemical methods are now finding increased acceptance over the bioassay methods (1, 2). Marzys (3) has described the application of spectrophotometric procedures for the determination of individual components in mixtures where the results are computed by solving simultaneous equations. Pernarowski, *et al.* (4), have described the application of spectrophotometric assay for the components of binary mixtures based on the location of isoabsorptive points. Elvidge and Peutrell (5) have described a (ΔE) spectrophotometric method which is based on a single wavelength and two solvents at different pH. The present paper describes a (ΔE) spectrophotometric method based on two wavelengths and one solvent for the determination, without prior separation, of chloramphenicol and tetracycline hydrochloride present in binary mixtures.

EXPERIMENTAL

Materials and Equipment.—Chloramphenicol U.S.P. (99.5%) from Carlo Erba, S.p.A., Milan, Italy, tetracycline hydrochloride U.S.P. (99.6%) from Messrs. Eigenmann & Veronelli, S.p.A., Milano, 0.1 *N* ammonium hydroxide, a Beckman spectrophotometer model G 2400 with DU photomultiplier combination G 4700 using hydrogen discharge tube, and 1-cm. silica cells were the materials and equipment employed in this study.

From a study of the spectral characteristic curves of chloramphenicol and tetracycline hydrochloride using water as the solvent, it was observed that by determining the absorptivities ($E^{1\%}_{1cm.}$) at 278 mμ and 360 mμ and using simultaneous equations, the composition of the binary mixture could be determined. However, this procedure involves relatively long calculations.

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Experiments were performed to determine the isoabsorptive wavelength using various solvents. Water was found unsuitable as a solvent for this purpose, and the use of 0.1 *N* acetic acid gave two isoabsorptive wavelengths, *viz.*, 282.5 mμ and 297.5 mμ. The slopes of curves at the former wavelength were more steep than those at the latter wavelength. Various mixtures of chloramphenicol and tetracycline hydrochloride were assayed by this method by measuring absorbances at 278 mμ, 297.5 mμ, and 360 mμ but the method lacked precision. The use of 0.1 *N* sodium hydroxide gave some interesting results. Chloramphenicol gave almost the same spectral characteristics as with 0.1 *N* acetic acid except for a slight depression of absorbance at the maxima. Tetracycline hydrochloride gave almost constant values of absorptivity in the spectral range of 230 mμ to 280 mμ. In this range, $E^{1\%}_{1\,cm.}$ for chloramphenicol registered a steep continuous increase up to 275 mμ, whereas those for tetracycline hydrochloride remained almost the same.

It was thus evident that chloramphenicol could be determined in the presence of tetracycline hydrochloride by making use of the "difference in absorptivity values" (ΔE) at two suitable wavelengths. The use of 0.1 *N* sodium hydroxide was found to be critical as regards time for ΔE measurements. Higher concentrations of alkali, such as 0.25 *N*

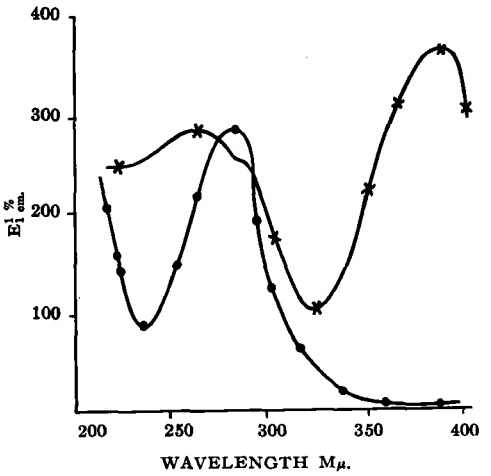

Fig. 1.—$E^{1\%}_{1\,cm.}$ curve for chloramphenicol (●—●), $E^{1\%}_{1\,cm.}$ curve for tetracycline hydrochloride (X—X).

TABLE I.—$E^{1\%}_{1cm.}$ OF CHLORAMPHENICOL AND TETRACYCLINE HYDROCHLORIDE IN SUITABLE RANGES OF WAVELENGTHS USING 0.1 *N* AMMONIUM HYDROXIDE

Wavelength, mμ	231	232	233	234	235	280	281	282	283	284
Chloramphenicol	110.5	107.5	102.5	97.5	97.5	290	290	292.5	290	285
Tetracycline hydrochloride	257.5	260	262.5	265	267.5	267.5	265	262.5	262.5	260

TABLE II.—PERCENTAGE RECOVERY OF CHLORAMPHENICOL AND TETRACYCLINE HYDROCHLORIDE FROM SYNTHETIC MIXTURES

Synthetic Mixtures		Found by the Method Described		Percentage Recovery	
Chloramphenicol, mcg./ml.	Tetracycline Hydrochloride, mcg./ml.	Chloramphenicol, mcg./ml.	Tetracycline Hydrochloride, mcg./ml.	Chloramphenicol	Tetracycline Hydrochloride
2	18	2.3	17.5	115	96.9
4	16	4.2	15.9	105	99.4
6	14	5.9	14	98.3	100
8	12	8.1	12	101	100
10	10	9.9	10.1	99	101
12	8	12.1	8.1	100.1	100.1
14	6	13.8	5.9	98.6	98.3
16	4	15.9	4	99.4	100
18	2	17.6	2.25	112.5	97.7

sodium hydroxide, were not critical in the determination of ΔE values for chloramphenicol. However, with this strength of sodium hydroxide the determination of tetracycline hydrochloride spectrophotometrically at 380 mμ requires strict adherence to time (6, 7). The use of 0.1 *N* ammonium hydroxide satisfied all requirements. The spectral characteristics of both chloramphenicol and tetracycline hydrochloride were almost the same as with 0.1 *N* sodium hydroxide. Furthermore, with ammonia, the normality of the solution and time for taking readings were not critical. The absorbance values remained almost the same for 3 hours.

Determination of ($\Delta E^{1\%}_{1cm.}$) for Chloramphenicol and $E^{1\%}_{1cm.}$ for Tetracycline Hydrochloride.—Solutions containing 20 mcg./ml. of tetracycline hydrochloride and chloramphenicol were prepared in 0.1 *N* ammonium hydroxide and spectrophotometric readings were taken in the ultraviolet range of 220 mμ to 400 mμ using 0.1 *N* ammonium hydroxide as blank (Fig. 1). For the determination of chloramphenicol, the most suitable wavelengths were 233 mμ and 282 mμ, and $\Delta E^{1\%}_{1cm.}$ was found to be 190 (Table I). For tetracycline hydrochloride, the most suitable wavelength was 380 mμ and $E^{1\%}_{1cm.}$ was 355.

Recovery Experiments.—Various mixtures containing a total of 20 mcg./ml. of chloramphenicol and tetracycline hydrochloride were prepared in 0.1 *N* ammonium hydroxide. Absorbances were measured at 233 mμ, 282 mμ, and 380 mμ using 0.1 *N* ammonium hydroxide as blank. Chloramphenicol was determined from the difference in absorptivities at 282 mμ and 233 mμ taking $\Delta E^{1\%}_{1cm.}$ as 190. Tetracycline hydrochloride was determined from the absorptivity at 380 mμ taking $E^{1\%}_{1cm.}$ as 355. The results are tabulated in Table II and show that the method gives very satisfactory results except at the two extremes.

Results.—The method was satisfactorily applied for the assay of commercial capsules of chloramphenicol 200 mg. and tetracycline hydrochloride 100 mg.[1] The usual excipients like lactose, amylum, and talcum were not found to interfere in the assay. The results compared very well with those of the usual method of determining chloramphenicol and tetracycline hydrochloride, after resolving the mixture into its components, by selective solvent extraction with ethyl acetate.

DISCUSSION

From Table II, it is evident that the method can safely be applied to the mixtures containing 20% and 80%. In practice, the majority of preparations will contain these two antibiotics within this range and hence the method is of great practical value. Again the method offers an advantage over the official spectrophotometric method for the determination of tetracycline hydrochloride (7) because strict adherence to time is not necessary when 0.1 *N* ammonium hydroxide is used as a solvent. The normality of ammonium hydroxide is not critical as it may range from 0.07 *N* to 0.13 *N* without affecting the results. Finally the ΔE value for chloramphenicol and $E^{1\%}_{1cm.}$ at 380 mμ value for tetracycline hydrochloride may vary slightly from instrument to instrument; hence these values must be ascertained on the individual instrument.

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[1] Marketed as Kemicycline capsules by Carlo Erba S.p.A., Italy.

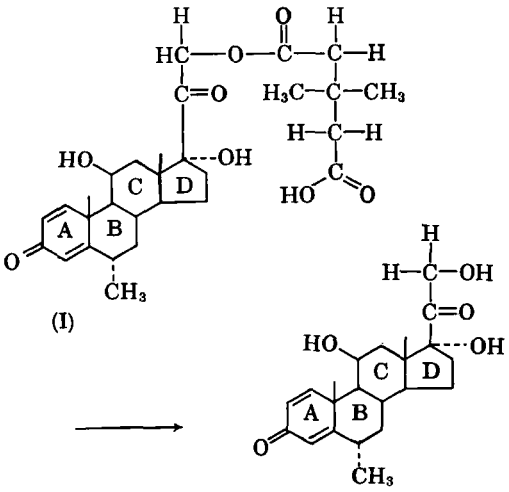
Colorimetric Procedure for Determination of Methylprednisolone Hemi-ββ′-dimethylglutarate and Methylprednisolone in Aqueous Solutions

By THOMAS CHULSKI and DONALD J. LAMB

An analytical procedure for the determination of methylprednisolone hemi-ββ′-dimethylglutarate and its hydrolysis product, methylprednisolone, was developed. The method involves separation of the steroids by extraction, followed by their colorimetric assay.

ONE APPROACH to the production of an aqueous pharmaceutical preparation of a cortical steriod is the use of the hemiesters of dicarboxylic acids. Of particular interest is the hemiester of methylprednisolone[1] and ββ′-dimethylglutarate (DMG).

The hydrolysis of methylprednisolone DMG (I) yields methylprednisolone (II) which is also physiologically active. Upon degradation of the dihydroxyacetone side chain, methylprednisolone would yield one or more neutral and acidic compounds which absorb radiation in the ultraviolet region of the spectrum (1).



(I)

(II)

Although ring A is photolabile (2), this mode of degradation is not important in pharmaceutical preparations if suitable precautions are taken to provide for protection from light (3).

Thus, in the stability testing of an aqueous pharmaceutical preparation of methylprednisolone DMG, assay methods are required that would be specific for I and II in the presence of each other and in the presence of degradation products. Ultraviolet absorption spectroscopy is not applicable, even in the absence of extraneous ultraviolet absorbing materials, as both active species have the same ultraviolet chromophore, *i.e.*, the A ring. Partitioning between water at a high pH and an immiscible solvent followed by ultraviolet spectroscopy is not applicable, as the neutral and acidic degradation products would interfere when present (1).

An assay procedure was developed that separated the two active compounds by extracting an aqueous solution at a high pH with chloroform. The chloroform phase was assayed for methylprednisolone using the blue tetrazolium procedure (4, 5). The aqueous phase was assayed for methylprednisolone DMG by acidifying, extracting with chloroform, and applying the method of Porter and Silber (6). Neither of the methods is affected by the presence of degradation products (4, 7).

EXPERIMENTAL

Determination of Partition Coefficient vs. pH.—Aqueous solutions containing 500 mcg. of methylprednisolone DMG per ml. were prepared using 0.5 *M* phosphate buffers. Two-milliliter volumes of these solutions were equilibrated with 25 ml. of water-saturated chloroform at room temperature. The layers were separated and the amount of steroid in each phase was determined by ultraviolet spectroscopy. Hydrolysis of the ester was assumed to be negligible during the equilibration period. The results listed in Table I indicate that essentially all of the hemiester remains in the aqueous layer at pH 10.0.

Color Development of Methylprednisolone Hemi-ββ′-dimethylglutarate and the Porter-Silber Re-

TABLE I.—PARTITION COEFFICIENT FOR METHYLPREDNISOLONE ββ′-DIMETHYLGLUTARATE SODIUM BETWEEN WATER AND CHLOROFORM AT VARIOUS pH'S AT ROOM TEMPERATURE

pH	$C_{organic}/C_{aqueous}$
7.8	0.643
9.0	0.040
10.0	0.01

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[1] Marketed as Medrol by The Upjohn Company, Kalamazoo, Mich.

agent.—To our knowledge, the applicability of the Porter-Silber reagent to steroidal hemiesters such as the ββ′-dimethylglutarate has not been reported in the literature. Hence, the formation of color by methylprednisolone DMG in the Porter-Silber procedure was studied as a function of time, and compared to methylprednisolone.

Two-milliliter volumes of a 1.43×10^{-4} *M* 3A alcoholic solution of methylprednisolone DMG were mixed with 4-ml. volumes of a dilute sulfuric acid solution of phenylhydrazine hydrochloride (see Reagents, below), and with 4 ml. of the dilute acid only. In the same manner, 2-ml. volumes of 3A alcohol were mixed with the two reagents to serve as blanks. The reaction mixtures were heated for various times, 20–60 minutes, in a water bath at 60°, and then cooled rapidly in cold water. The absorbance was measured at 415 mμ in a 10-mm. cell with the appropriate 3A alcohol blank in the reference cell in each instance. The Porter-Silber color was determined by difference, *i.e.*, $A_{steroid} = A_{reagent\ sample} - A_{acid\ sample}$. A 1.43×10^{-4} *M* solution of methylprednisolone was handled in the same manner.

The data are presented graphically in Fig. 1 Although the color development with methylprednisolone DMG is not complete after 60 minutes at 60°, the rate of change is small enough, about 4%, that heating at 60° for 60 minutes would be satisfactory as an analytical procedure.

METHOD

Reagents.—*pH 10.0 buffer.* Seventy-one grams of anhydrous sodium dibasic phosphate, Mallinckrodt A.R. grade, was dissolved in 1000 ml. of water. The pH was adjusted to 10.0 with 1.0 *M* sodium hydroxide solution; *ca.* 30 ml. was required.

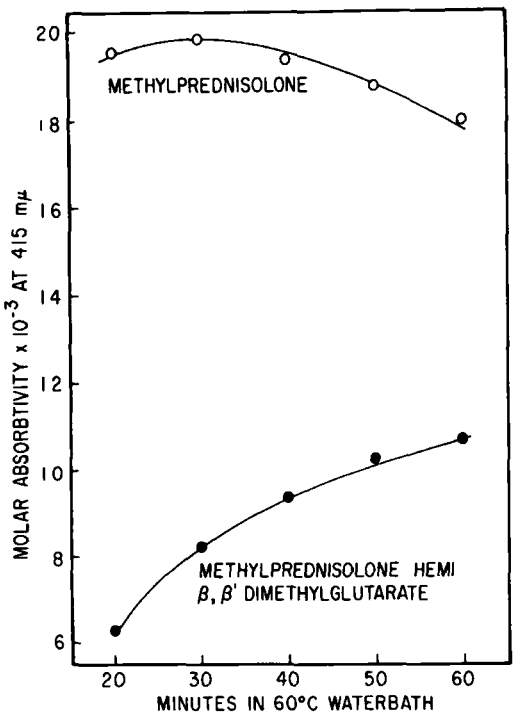

Fig. 1.—Development of the Porter-Silber color with methylprednisolone and with methylprednisolone hemi-ββ′-dimethylglutarate.

Blue tetrazolium solution. One hundred twenty-five milligrams of blue tetrazolium[2] was dissolved in 3A alcohol and diluted to 50 ml.

Tetramethylammonium hydroxide solution. Five milliliters of a 10% aqueous solution[3] was diluted to 50 ml. with 3A alcohol. These two solutions were usable for three days if refrigerated when not in use.

Sulfuric acid solution. Three hundred and ten milliliters of concentrated sulfuric acid and 190 ml. of water were mixed.

Phenylhydrazine hydrochloride solution. Phenylhydrazine hydrochloride[3] was recrystallized twice from 3A alcohol. One hundred fifty milligrams of the pure white material was dissolved in the dilute sulfuric acid and diluted to 100 ml. This reagent was prepared fresh daily.

Chloroform. Reagent grade.

Procedure.—Two-milliliter aliquots of aqueous samples containing 50–500 mcg./ml. of sodium methylprednisolone DMG and also of a standard solution were diluted with 3 ml. of pH 10 buffer and extracted with two 25-ml. portions of chloroform. The solvent was removed from the combined chloroform extracts under reduced pressure at room temperature. The dry residue was dissolved in 4 ml. of 3A alcohol. One milliliter of blue tetrazolium solution was added, followed by 0.5 ml. of tetramethylammonium hydroxide solution, and timing started. Forty-five ± 1 minutes later, the color development was quenched and the color was stabilized by adding 0.5 ml. of glacial acetic acid (8). The absorbance was measured at 525 mμ in a 10-mm. cell with 3A alcohol in the reference cell. A chloroform extract of 2 ml. of water treated in the same manner as the sample extract served as a reagent blank. Its absorbance was subtracted from that of the sample.

Beer's law was followed up to 12.5 mcg. methylprednisolone per ml. final volume.

The sample, after the removal of the methylprednisolone, and the water blank were acidified with 1 ml. of 6 *M* hydrochloric acid and extracted with 25 ml. of chloroform. A 10-ml. aliquot of the chloroform extract was taken to dryness. The residue was dissolved in 5 ml. of 3A alcohol. Two 2-ml. aliquots were transferred to glass-stoppered test tubes. To one of the aliquots was added 4 ml. of the phenylhydrazine solution; to the other was added 4 ml. of the dilute sulfuric acid. Both were heated for 60 minutes in a water bath at 60° and then cooled rapidly in cold water. The absorbance was measured at 415 mμ with the appropriate blank in the reference cell. The absorbance, due to the steroid, was obtained by difference, *i.e.*, $A_{steroid} = A_{reagent\ sample} - A_{acid\ sample}$.

The molar absorptivity for methylprednisolone ββ′-dimethylglutarate sodium was found to be approximately 10,000. Beer's law followed up to 53 mcg. per ml. final volume.

DISCUSSION

The results of the partition study indicate that a small amount of hemiester accompanies the extrac-

[2] Certified reagent marketed by Fisher Scientific Co.
[3] Marketed by Eastman Organic Chemicals.

tion of methylprednisolone. The molar absorptivity of the hemiester in the blue tetrazolium procedure is 513, while that of the steroid alcohol is 29,190. Therefore, the effect of minor amounts of the hemiester on the assay of methylprednisolone would be negligible.

From Fig. 1 it is seen that on a molar basis methylprednisolone DMG is 61% as chromogenic as methylprednisolone. It is interesting to note that the chromogenicities of hydrocortisone acetate and cyclopentylpropionate in the Porter-Silber reaction are equal to that of hydrocortisone (9).

Marcus (10) determined hydrocortisone phosphate with the Porter-Silber procedure by heating for 2 hours at 60°. He did not state the chromogenicity of the phosphate ester relative to hydrocortisone under the same conditions.

The sample of steroid ester used in this investigation was found by papergram analysis to contain approximately 2% of the steroid alcohol. The necessity of purifying the material was circumvented by a synthetic sample approach. Known amounts of methylprednisolone were added to the chloroform used to extract a standard volume of hemiester solution, and the samples were assayed using the procedure described. The results of these assays are listed in Table II. A plot of micrograms of methylprednisolone added *vs.* micrograms found yielded a straight line, as shown in Fig. 2. The intercept of the line indicates that 2.63 ± 0.04% steroid alcohol was present in the sample of hemiester.

The average variation of individual results from the average of duplicate results was 2.5% for the methylprednisolone DMG in simple systems. The standard deviation for the methylprednisolone assay was ±1.3%.

The procedure was applied successfully to pharmaceutical formulations.

TABLE II.—THE ADDITION OF KNOWN AMOUNTS OF METHYLPREDNISOLONE TO METHYLPREDNISOLONE ββ′-DIMETHYLGLUTARATE KNOWN TO CONTAIN SOME METHYLPREDNISOLONE[a]

Methylprednisolone, mcg.	
Added	Found
0	26.0, 26.1, 27.6, 27.6
10.0	33.7, 35.3, 36.9, 37.6
25.0	49.7, 49.9, 50.0, 52.2
40.0	65.5, 66.1, 67.2, 67.6, 68.0
50.0	76.0, 76.2, 76.4, 76.7, 76.8

[a] Slope of the least squares line = 1.001 ± 0.013 (S.D.). Intercept 26.26 ± 0.42. Since 1000 mcg. of hemiester was taken, % of methylprednisolone present = 2.63 ± 0.04.

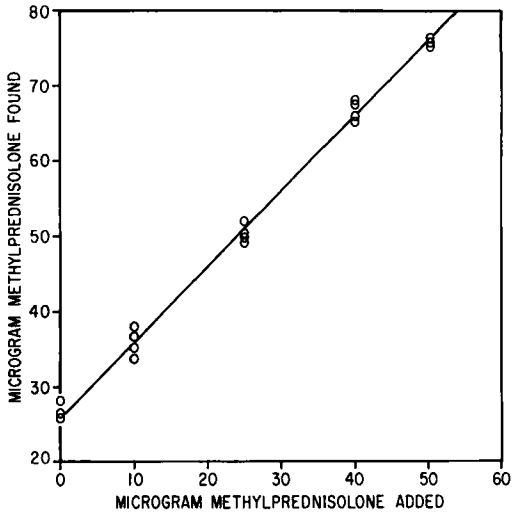

Fig. 2.—The addition of known amounts of methylprednisolone to methylprednisolone ββ′-dimethylglutarate known to contain some methylprednisolone.

SUMMARY AND CONCLUSIONS

A colorimetric method is described for determining methylprednisolone ββ′-dimethylglutarate and methylprednisolone in aqueous solution in the presence of each other and in the presence of degradation products.

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Effect of Monoamino Oxidase Inhibitors on Audiogenic Seizures

By N. PLOTNIKOFF, J. HUANG, and P. HAVENS

This study attempts to demonstrate that monoamino oxidase inhibitors possess anticonvulsant activity as evaluated by their effects on audiogenic seizures in mice.

A RECENT STUDY of iproniazid and pheniprazine in rats indicated that the two agents inhibit the tonic extensor component of convulsions induced by electroshock (1). The present study is concerned with the effects of several inhibitors on audiogenic seizures in mice.

EXPERIMENTAL

Methods.—The apparatus employed in the study has been described in earlier reports by Plotnikoff (2, 3). Essentially, the apparatus consisted of a metal auditory-test chamber placed inside a sound-deadened plywood box. The sound source consisted of two common doorbells attached to the inner wall of the metal test chamber. The test procedure consisted of placing a single animal inside the metal chamber and exposing it to auditory stimulation for 1 minute. The sole criterion used to measure protection in this study was the presence or absence of tonic extension. Each drug was tested at three or more dose levels with ten mice tested per dose. The dose that reduced tonic extension responses to 50% of the control values (PD_{50}) and its standard error were estimated by the technique of Miller and Tainter (4). The drugs were administered by the intraperitoneal route. The animals used for this study were random bred male Swiss mice susceptible to audiogenic seizures.[1]

Studies on convulsions induced by electroshock were based on the maximal electroshock seizure test (M.E.S.) in mice reported by Swinyard, *et al.* (5), for maximal electroshock (50 *mA*). Normal albino Swiss mice (18–22 Gm.) nonsusceptible to audiogenic seizures were used for the M.E.S. test.

Results.—The anticonvulsant effects of several monoamino oxidase inhibitors are shown in Table I. The first agent to be reported upon is iproniazid which was shown to have an anticonvulsant effect (inhibition of both tonus and clonus) 30 minutes after administration in a dose range from 130 to 200 mg./Kg. The protective dose in 50% of the animals (PD_{50}) was found to be 163 ± 8.5 mg./Kg.

The second agent, tranylcypromine, was found to have anticonvulsant activity (inhibition of tonic extension) 1 hour after administration in a dose range from 10 to 40 mg./Kg. The protective dose in 50% of the animals was found to be 16.5 ± 2.2 mg./Kg.

The third agent, pheniprazine, was found to be active (inhibition of tonic extension) 16 hours after administration in a dose range from 5 to 20 mg./Kg. The protective dose in 50% of the animals was found to be 11.0 ± 1.6 mg./Kg.

Etryptamine was found to be the most active agent against audiogenic seizures (inhibition of tonic extension), with a PD_{50} of 4.1 ± 0.6 mg./Kg. It was tested in a range from 1.25 to 20.0 mg./Kg. 2 hours after being administered.

Phenelzine also prevented tonic extension with a PD_{50} dose of 19.0 ± 3.0 mg./Kg., and was tested in a range from 15 to 60 mg./Kg.

Nialamide exerted protection against tonic extension and was found to have a PD_{50} dose of 8.7 ± 1.1 mg./Kg. and was tested in a range from 5 to 40 mg./Kg.

Comparative anticonvulsant studies were carried out in normal mice (not susceptible to audiogenic seizures) by maximal electroshock. The results are shown in Table II. Iproniazid was evaluated over a time period of 15 minutes to 8 hours after injection and found to be ineffective in preventing tonic extension. Tranylcypromine and pheniprazine exerted partial protection against tonic extension only at high doses. Phenelzine and nialamide were also found to be ineffective in preventing tonic extension. Mortality following maximal electroshock was not significantly different from that found in controls. Etryptamine exterted significant protection against tonic extension in a dose range from 5 to 40 mg./Kg. The PD_{50} was estimated to be 13.5 ± 1.8 mg./Kg.

DISCUSSION

The present study raises several questions regarding the efficacy of comparing anticonvulsant effects of drugs on seizures induced by audiogenic stimuli and electroshock. Most of the animals susceptible to audiogenic seizures are between 21 and 25 days of age, whereas mice used for electroshock are between 25 and 30 days of age. Thus, audiogenic-seizure-susceptible mice may be more sensitive to the effects of various drugs because of their age. A similar observation with known anticonvulsants was recently

TABLE I.—EFFECT OF MONOAMINO OXIDASE INHIBITORS ON AUDIOGENIC SEIZURES IN MICE

Drug	Time Tested After Drug Administration, hr.	Protective Dose 50%, mg./Kg.
Iproniazid	1/2	163.0 ± 8.5
Tranylcypromine	1	16.5 ± 2.2
Pheniprazine	16	11.0 ± 1.6
Etryptamine	2	4.1 ± 0.6
Phenelzine	2	19.0 ± 3.0
Nialamide	2	8.7 ± 1.1

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[1] Animal suppliers: Flora O'Grady, 2336 Gunther Avenue, New York, N. Y., and Simonsen Laboratories, Day Road, Gilroy, Calif.

TABLE II.—EFFECT OF MONOAMINO OXIDASE INHIBITORS ON CONVULSIONS INDUCED BY ELECTROSHOCK IN MICE

Drug	Time Tested After Drug Administration	Dose, mg./Kg.	Number with Tonic Extension/Number Tested	Mortality
Iproniazid	Controls		10/10	3/10
	15 min.	400	10/10	4/10
	30 min.	400	10/10	2/10
	1 hr.	400	10/10	0/10
	2 hr.	400	10/10	1/10
	4 hr.	263	10/10	1/10
	6 hr.	263	10/10	3/10
	8 hr.	263	10/10	0/10
	15 hr.	240	9/10	1/10
	15 hr.	288	10/10	2/10
	15 hr.	345	9/10	0/10
	15 hr.	414	9/10	3/10
Tranylcypromine	Controls		18/19	2/19
	1 hr.	10	20/20	5/20
	1 hr.	20	16/17	6/17
	1 hr.	40	9/23	7/18
Pheniprazine	Controls		20/20	5/20
	2 hr.	40	10/10	1/10
	4 hr.	40	10/10	1/10
	6 hr.	15	9/10	2/10
	6 hr.	30	9/10	6/10
	6 hr.	60	7/10	1/10
	8 hr.	40	6/10	1/10
	10 hr.	40	6/10	2/10
Etryptamine	Controls		10/10	3/10
	2 hr.	5	9/10	5/10
	2 hr.	10	8/10	3/10
	2 hr.	20	2/10	3/10
	2 hr.	40	0/10	3/10
Phenelzine	Controls		10/10	4/10
	30 min.	20	10/10	4/10
	1 hr.	20	10/10	0/10
	2 hr.	20	10/10	4/10
	4 hr.	20	10/10	2/10
Nialamide	Controls		10/10	1/10
	2 hr.	25	8/10	2/10
	2 hr.	50	10/10	2/10
	2 hr.	100	10/10	1/10

reported by Swinyard (6). Although both stimuli (electroshock and auditory) produce the very same kind of convulsion (clonic-tonic), there may be a difference in stimulus spread in the central nervous system. Thus, the anticonvulsant effects of monoamino oxidase inhibitors in mice with audiogenic seizures and on normal animals may vary considerably. The latter were employed for evaluation of agents against convulsions induced by maximal electroshock. Nevertheless, such comparisons are useful for studying anticonvulsant drug responses. In the present study, the monoamino oxidase inhibitors did not exert significant anticonvulsant effects in mice experiencing convulsions induced by maximal electroshock. An exception was etryptamine which protected animals from tonic extension. The anticonvulsant mechanisms involved may be related to levels of central amines, although recently Lehmann (7) reported that there was not a complete parallelism between anticonvulsant effects and serotonin brain levels. Perhaps a more complete correlation can be established between other central intermediates such as norepinephrine, pyridoxal 5-phosphate, dihydroxyphenylalanine, or dopadecarboxylase.

SUMMARY

Mice susceptible to audiogenic seizures and normal mice were employed to evaluate the anticonvulsant activity of monoamino oxidase inhibitors. This study has demonstrated that tranylcypromine, pheniprazine, etryptamine, phenelzine, and nialamide inhibit the tonic extensor component of audiogenic seizures in mice. Iproniazid was found to inhibit both the tonic and clonic components of audiogenic seizures in mice. With the exception of etryptamine, all of the inhibitors were inactive in preventing convulsions induced by electroshock (M.E.S. test).

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Spectrophotometric Determination of Cholesterol in Wool Fat Using the Zak Reaction

By BLAKE F. PUTNEY and ANTHONY N. PARUTA

In an effort to overcome some of the shortcomings of previous methods for determination of cholesterol in wool fat, the Zak reaction has been studied. A procedure is presented in which cholesterol is separated as its digitonide with subsequent development of color by the addition of ferric ammonium chloride and sulfuric acid. The method is reproducible and appears to be superior to other methods now in use.

THE spectrophotometric methods currently in use for the determination of cholesterol in wool fat are for the most part based on the Liebermann-Burchard reaction (1–3). The use of this reaction as a quantitative measure of cholesterol, however, leaves much to be desired due to the dependence of the reaction on temperature, time of color development, and stability of the reagent. The ferric chloride reaction proposed by Zlatkis, Zak, and Boyle (4) for the determination of cholesterol has been shown to be a reliable method for determination of blood cholesterol. Later publications by Zak (5–7) have set forth improvements in the original procedure.

The color produced by the Zak reagent with cholesterol has been found to be reproducible without the necessity for temperature control. The color reaches a maximum in about 10 minutes and is sufficiently stable to be read in the period of 10–50 minutes after mixing. The reagent, composed of ferric ammonium chloride ($FeCl_3 \cdot 2NH_4Cl \cdot H_2O$) in 80% acetic acid is stable for periods up to six months. This new method has, therefore, overcome most of the troublesome aspects of the procedure in which the Liebermann-Burchard reaction is used.

It was the purpose of this research to determine if the Zak reaction could be utilized in the analysis of wool fat and wool fat-containing products for cholesterol content. It appears to the authors that the reaction can be used for this purpose.

EXPERIMENTAL

Equipment.—The Beckman DK-2 recording spectrophotometer was used for determination of the various spectra. The routine colorimetric analyses were run on a Coleman Universal spectrophotometer, using square cuvettes of 1.3 cm. light path.

Materials.—Cholesterol U.S.P.,[1] after crystallization from alcohol-water and drying at 80°, had m.p. 148–149°, $[\alpha]_{25}^{D}$ ($CHCl_3$) −38.4°. The lanosterol[2] used had a m.p. of 137-138°. Wool fat U.S.P., wool fat unsaponifiable, and a liquid wool fat fraction were used in the sample analyses.[3] All other materials used were analytical reagent grade, except ethanol and potassium hydroxide, which were U.S.P., and acetone and aluminum chloride, which were N.F.

Preparation of Solutions.—Stock solutions for use in the experimental procedures were accurately prepared as shown in Table I.

TABLE I.—PREPARATION OF SOLUTIONS

Solute	Gm./100 ml.	Solvent
Ferric ammon. chlor.	2.12	80% Acetic acid
Digitonin	1.00	50% Ethanol
Cholesterol	0.20–0.30	Ethanol
Lanosterol	0.10	Ethanol
Wool fat unsaponifiable	0.20	Ethanol
Wool fat solution I[a]	0.15–0.40	20% Dioxane, 20% acetone in ethanol
Wool fat solution II[a]	0.050	Acetic acid

[a] *Wool fat or liquid wool fat fraction. Heat was necessary to bring about solution. Solution I was used for saponification and precipitation; solution II was used for direct color analysis.*

Procedure: Preliminary Saponification.—An amount of wool fat solution I (Table I) equivalent to 8–12 mg. of wool fat was placed in a 15-ml. centrifuge tube and ethanol added to make 5 ml. (if necessary). To the tube was added 0.2 ml. of 50% aqueous potassium hydroxide and one drop of phenolphthalein T.S. The tube was boiled in a water bath for 5 minutes, loosely stoppered, and heated on a sand bath in an oven at 65° for 3 hours. At the end of the heating period, 0.5 ml. of water was added and the mixture was neutralized by dropwise addition of 10% hydrochloric acid. This solution was then treated with digitonin as further described.

Digitonin Precipitation.—The digitonin precipitation was carried out on the neutralized solution resulting from the saponification contained in a 15-ml. centrifuge tube. Where saponification was not necessary, an amount of stock solution (Table I) equivalent to 2–3 mg. of cholesterol or 6–8 mg. of wool fat unsaponifiable was placed in 15-ml. centrifuge tubes and ethanol added to make 5 ml. (if needed).

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[1] Obtained from American Cholesterol Products Inc., Edison, N. J.

[2] Obtained from Wed. D. S. van Schuppen and Zoon, Holland, through the courtesy of N. I. Malmstrom Co., Brooklyn, N. Y.

[3] Obtained from the Malmstrom Chemical Corp., Newark, N. J.

Two drops of 30% aqueous aluminum chloride and 1 ml. of 1% digitonin solution were added to each tube. The tube was gently boiled in a water bath for 5 minutes and then allowed to stand at 20° for 2 hours. The mixture was centrifuged at 2,800 r.p.m. for 5 minutes and the supernatant liquid decanted. The precipitate was washed twice with 5 ml. of acetone, the precipitate being broken up each time with a fine glass rod, and then centrifuged. After the final spinning, the supernatant liquid was decanted and the precipitate allowed to dry. The residue was then dissolved in warm acetic acid and brought to volume in a 10-ml. volumetric flask.

Colorimetric Analysis.—For colorimetric analysis, 1 ml. of the acetic acid solution of the digitonide was used. The stock solutions (Table I) were diluted with acetic acid so that they contained 0.2–0.3 mg. of cholesterol or lanosterol or 0.4–0.6 mg. of wool fat unsaponifiable per ml.; 1 ml. of each was used for colorimetric analysis. Three milliliters of wool fat solution II (Table I) was used for analysis.

The specified quantity of the respective solutions was pipetted into 18 × 150-mm. tubes and acetic acid added from a buret to make 6 ml.; 0.40 ml. of ferric ammonium chloride solution was added, and the contents mixed. Four milliliters of concentrated sulfuric acid was added slowly down the side of the tube to form a layer under the acetic acid solution. The contents were then mixed thoroughly on a Vortex Jr. mixer and allowed to cool for at least 10 minutes. The absorbance of the solution at 560 mμ was taken in the period of 10–25 minutes after adding sulfuric acid. The blank consisted of 6 ml. of acetic acid, 0.4 ml. of ferric ammonium chloride, and 4 ml. of sulfuric acid, which had been mixed as before.

DISCUSSION

Ideally, it would be desirable to determine the amount of cholesterol directly, without prior treatment or separation of the cholesterol from wool fat. This would necessitate the determination of the effect of the reagent on the other constituents of wool fat which might give a color with the reagent. Lanosterol, which is the chief component of the triterpene fraction of wool fat and reported to be present in wool fat to the extent of about 15% (3), was used as a measure of this effect. On determination of the spectra of lanosterol and cholesterol (Fig. 1), it was found that a maximum of absorbance occurs for lanosterol at 490 mμ. From this plot of the absorptivities it is seen that there would be considerable interference of one constituent with the other at that wavelength. This is a situation which was found to make precise analysis of 1:1 mixtures of these components difficult to obtain.

Since in wool fat most of the cholesterol is present as fatty acid esters, the effect of this esterification would also have to be considered. Assous and Girard (8) have shown that the fatty acid esters of cholesterol do not exhibit the same rate of color development as does free cholesterol. These workers added the samples for analysis and the reagent to previously mixed and cooled acids, and allowed the reaction to take place at room temperature. Under these conditions the fatty acid esters of cholesterol did not develop any appreciable color in 1 hour, while the color developed by free cholesterol became constant after about 30 minutes.

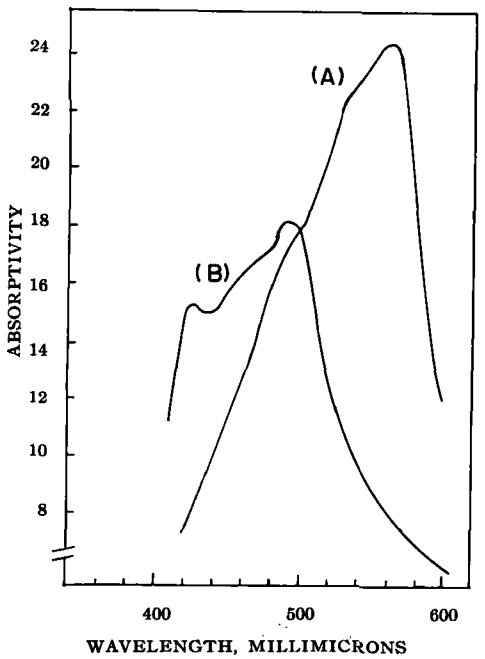

Fig. 1.—The spectra of cholesterol and lanosterol on treatment with the Zak reagent. *A*, cholesterol; *B*, lanosterol.

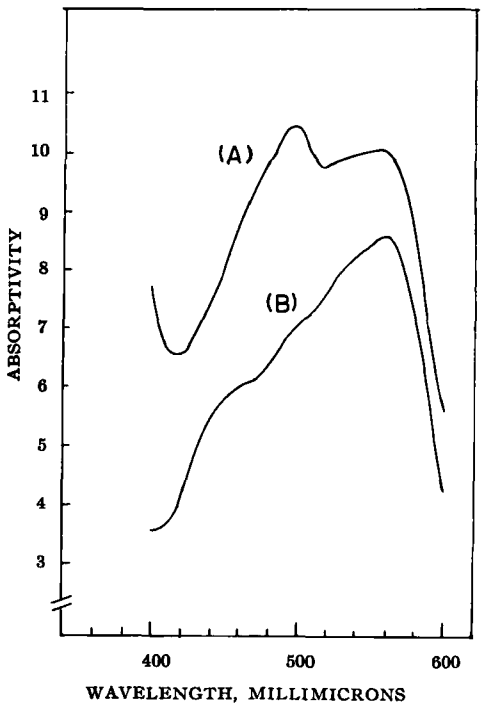

Fig. 2.—The spectra of wool fat unsaponifiable reacted directly with Zak reagent and after precipitation, with digitonin. The absorptivities were calculated using the concentration of wool fat unsaponifiable in the color solutions. *A*, reacted directly; *B*, after digitonin precipitation.

In the procedure used in the present work, elevation of temperature takes place on mixing the acetic acid solution of the cholesterol and reagent with sulfuric acid. On studying the stability of the color complex under the conditions used here, it was found that the absorbance of the color complex of untreated wool fat (containing the fatty acid esters) reached a maximum in about 60–70 minutes, while the color complex of cholesterol or cholesterol digitonide reached a maximum in about 10 minutes and remained the same or decreased slightly during the period of 1 hour after addition of the acid. This indicates that the fatty acid esters of cholesterol also react more slowly under the present conditions than does cholesterol. This is contrary to the situation with the Liebermann-Burchard reaction, in which the esters of cholesterol react more rapidly.

In sample analyses carried out on wool fat directly, the amount of cholesterol found was somewhat less than that found when wool fat was first saponified and the free cholesterol precipitated by digitonin before color development (Table II). This may be partially explained by the difference in the rate of reaction of the fatty acid esters of cholesterol, since more compatible results were obtained when the wool fat unsaponifiable fraction was analyzed by direct color analysis or by the precipitation technique (Table II).

From a practical standpoint also, it would be desirable to have a technique which would be useful in the determination of the amount of wool fat in pharmaceutical and cosmetic products. It was concluded that the best way to separate the color-producing moiety from such mixtures was by saponification and precipitation of the free cholesterol with digitonin, before color development. The saponification procedure suggested by Luddy, *et al.* (1), was used and the saponified mixture was treated directly with digitonin to precipitate the cholesterol.

It was found that lanosterol did not give a precipitate with digitonin under the conditions utilized here and so it was not necessary to calculate for lanosterol in this procedure. From an examination of the spectra of wool fat unsaponifiable, treated and untreated with digitonin (Fig. 2) it appears that there is no appreciable increase in the absorption of the treated sample at 490 mμ where a peak would be expected if there were lanosterol present.

Cholesterol digitonide was shown to have a slightly higher absorptivity than free cholesterol when treated with the iron reagent (Fig. 3). This difference can probably be accounted for by the absorbance of digitonin when treated with the reagent, since as shown by the spectrum of digitonin, there is a small amount of absorbance at the primary wavelength of cholesterol, 560 mμ.

The optimum ratio of sulfuric acid to acetic acid (2:3) in the reaction mixtures was determined by Zak (9). This ratio appears to be necessary for the proper formation of the color complex due to the amount of heat liberated on dilution of the sulfuric acid. Immediately after mixing the solutions, an amber color is observed, which changes through violet to a reddish-violet color after about 5 minutes. In time studies on the color complexes of both cholesterol and its digitonide, there was noted a decrease in absorbance of from 2–3% during the period of 10–50 minutes after mixing the solutions.

Brown and co-workers (10) have investigated the use of several agents to facilitate the flocculation of cholesterol digitonide in blood serum studies. These workers reported that aluminum chloride was very effective for this purpose. In the studies on wool fat, it was found that separation and washing of the digitonide precipitate was more easily carried out in the presence of aluminum chloride.

The viscosity of the solution resulting from the mixture of sulfuric acid and acetic acid was a source of some difficulty, particularly with respect to adequate mixing of the two acids. The use of the Vortex Jr. mixer seemed to solve this problem to a considerable extent. Occasionally in the mixing process air bubbles would become entrapped in the solution, which would usually be dissipated by the time the solution had cooled.

TABLE II.—COMPARISON OF RESULTS OBTAINED ON DETERMINATION OF CHOLESTEROL BY DIRECT TREATMENT WITH THE ZAK REAGENT AND AFTER SAPONIFICATION AND PRECIPITATION WITH DIGITONIN

Sample	Direct Method[a]	Sapon. and Pptn. Method[b]
IIIb-L	13.5[c]	19.1[c]
IVb-W	11.6	20.7
W.F. unsap.	32.5	35.4

[a] Acetic acid solutions of wool fat or wool fat unsaponifiable were analyzed colorimetrically. [b] Acetic acid solutions of the digitonide obtained from wool fat unsaponifiable or wool fat after saponification and precipitation were analyzed. [c] Gm. of cholesterol found per 100 Gm. of wool fat or wool fat unsaponifiable taken for analysis.

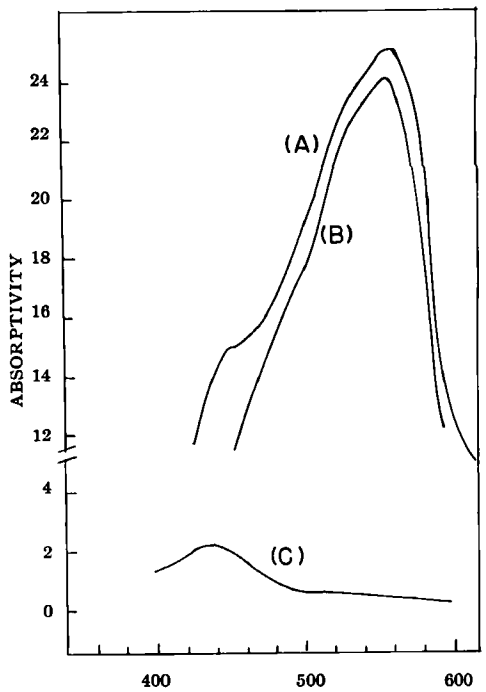

Fig. 3.—The spectra of cholesterol, cholesterol digitonide, and digitonin after treatment with the Zak reagent. The absorptivities were calculated using the concentration of the respective substances in the color solutions. *A*, cholesterol digitonide; *B*, cholesterol; *C*, digitonin.

RESULTS

The preliminary tests performed showed that the color complex formed with both cholesterol and its digitonide obeyed Beer's law (Fig. 4). This shows also the slightly greater absorbance of cholesterol digitonide than free cholesterol, as mentioned earlier.

The precision of the whole analytical procedure for determination of cholesterol in wool fat and its derivatives is shown in Table III. The difference of cholesterol content for different lots of wool fat or wool fat derivatives is also shown. However, within each lot of wool fat tested, good reproducibility was obtained with a maximum deviation from the mean of 6.8%. The average difference from the mean of all lots tested was 2.7% with a standard deviation from the mean of 3.3%. The calculations for the determination of cholesterol content were done by the use of the absorptivity of cholesterol digitonide, which had previously been determined.

The procedure as described requires some 6–7 hours to run, but does not require constant attention during the entire period. The periods of time given are for the most part minimum periods which were found to be necessary for completion of the respective reactions. The lapse of time between the addition of the sulfuric acid and the reading of the absorbance of the solution is not critical, since the readings in the analyses are taken in the period of 10–25 minutes after mixing the solutions.

TABLE III.—RESULTS OF ANALYSIS OF WOOL FAT AND WOOL FAT DERIVATIVES

Sample of Wool Fat	Chol. Found[a,b]	Mean Chol. Found[a]	% Difference from Mean
Ia-L	16.5	16.4	0.6
	16.5		0.6
	16.5		0.6
	16.2		1.2
Ib-L	16.0	16.4	2.4
	16.3		0.6
	16.8		2.4
IIa-L	19.6	18.9	3.7
	18.4		2.6
	18.7		1.0
IIb-L	18.0	17.4	3.4
	16.3		6.3
	16.9		2.9
	17.1		1.7
	18.5		6.3
IIIa-L	19.8	19.2	3.1
	18.5		3.6
IIIb-L	19.6	19.1	2.6
	20.4		6.8
	18.2		4.7
	18.2		4.7
IVa-W	21.7	20.8	4.3
	20.9		0.5
	20.2		2.9
	19.9		4.3
	21.3		2.4
	20.5		1.4
IVb-W	20.6	20.7	0.5
	21.0		1.4
	21.0		1.4
	20.3		1.9
Av. difference from mean		2.7%	
S.D. from mean (N = 31)		3.3%	

[a] Gm. cholesterol found per 100 Gm. of wool fat taken for analysis. [b] Each number represents the average obtained from color analysis of duplicate samples of acetic acid solutions of the digitonide after saponification and precipitation.

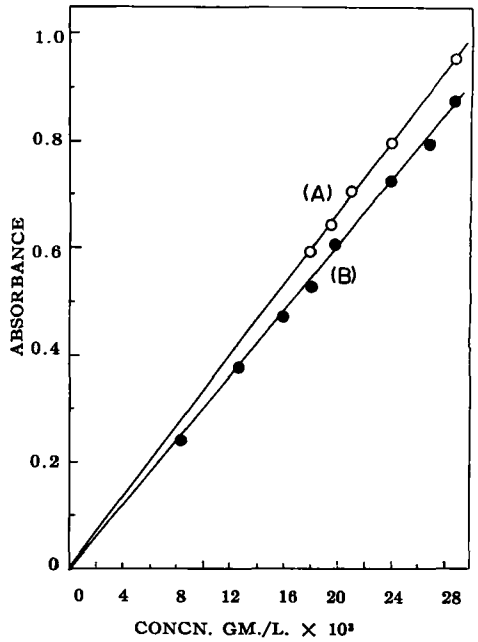

Fig. 4.—Beer's law plot of cholesterol and its digitonide using the Coleman spectrophotometer with 1.3-cm. cuvettes. *A*, cholesterol digitonide; *B*, cholesterol.

SUMMARY

A procedure is presented which is reproducible, which requires a minimum of control of conditions, and which constitutes a great improvement over previous methods in use. The reaction is quite sensitive; the analysis can be carried out using as little as 5 ml. of a mixture containing as little as 0.2% of wool fat. The reagent in use is stable for extended periods and no elaborate equipment is necessary. The method should lend itself to the analysis of many different types of pharmaceutical and cosmetic products containing wool fat or the many wool fat derivatives presently on the market.

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Comparative Evaluation of Tablet Formulations Prepared from Conventionally-Processed and Spray-Dried Lactose

By WILLIAM C. GUNSEL and LEON LACHMAN

Four tablet formulations made from two types of lactose were studied. The granulations were compared for particle size distribution, flow properties, and moisture content. The tablets made from them were evaluated for hardness, friability, disintegration time, weight control, and color development at room temperature, 40°, 50°, and 60° for 12 weeks. In general, it was found that spray-dried lactose produced harder, less friable tablets which, however, were more susceptible to color development following storage at elevated temperatures.

SINCE 1956, lactose suitable for use in tablets has been available in two types, depending upon the final manufacturing step. In the manufacture of lactose, the whey of milk is placed in vats where it is heated with dilute hydrochloric acid to coagulate the protein. The acidity is then neutralized with lime and the protein removed by filtration. The liquid portion is evaporated to produce a thick syrup containing 60–70% solids. Upon standing, the syrup crystallizes. After centrifuging the crystals are washed with water. This is crude lactose. The crude is dissolved in tap water at 180 to 200°F. and treated with carbon to decolorize it. It is again filtered and then sterilized by maintaining it at 210°F. for 25 to 35 minutes. It is reconcentrated to 60% solids in vats which then contain 10,000 to 14,000 pounds dry weight. It is at this point that the method of manufacture differentiates the two kinds of lactose discussed in this paper (1, 2). That which will be called conventionally-processed lactose is obtained by centrifuging first and then drying; the second kind is pumped into a spray dryer and becomes spray-dried lactose.

Spray-dried lactose contains about 8% of amorphous material; this is actually dried syrup which has not crystallized. Because the mother liquor becomes a part of the final material, spray-dried lactose contains larger amounts of impurities than the centrifuged product. Thus, it has about five times as much ash and protein, twice the heavy metals and "other sugars," and ten times the lipids (3).

In mesh size, spray-dried lactose has a small portion less than 100; over 94% passes through 100 mesh and 27 to 44% is finer than 200. Conventionally-processed lactose may be obtained in several mesh sizes. This article is mainly concerned with the fine material all of which passes through 100 and 40–50% through 200 mesh. The spray-dried lactose flows readily while the conventionally-processed has poor flow, except in the coarser mesh sizes.

Although spray-dried lactose is now also widely used in tablet formulations, literature about its tableting properties has not appeared in scientific journals. Accordingly, this study was undertaken to determine how the two kinds of lactose compared with each other as the major excipient in several representative tablet formulations.

The following aspects were evaluated: (*a*) particle size distribution in the granulations; (*b*) flow rate and angle of repose of the granulations; (*c*) changes in disintegration time, hardness, friability, and color, for several representative tablet formulations after storage at room temperature, 40°, 50°, and 60° (tests were made at 0, 4, 8, and 12 weeks); and (*d*) uniformity of tablet weight.

EXPERIMENTAL

Formulations

The first formulation (4) examined had the following composition

FORMULATION I

Lactose U.S.P.	88.5%
Tragacanth U.S.P.	2.0%
Polyethylene glycol 6000	4.0%
Confectioners sugar	2.0%
Talcum U.S.P.	3.0%
Magnesium stearate U.S.P.	0.5%
3A Alcohol 50%	*q.s.*

The polyethylene glycol was dissolved in the 50% alcohol and added to the mixture of the other components. The moist mass was passed through a No. 10 hand screen and dried with circulating, dehumidified air for a minimum of 16 hours. The granules were broken on a No. 16 hand screen and were then ready for compression.

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FORMULATION II

Lactose U.S.P.	94.5%
Cornstarch (dried)	4.5%
Stearic acid powder U.S.P.	1.0%
Purified water	*q.s.*

The lactose, stearic acid, and part of the starch were mixed together; the rest of the starch was converted to a paste with the water and used to wet down the powders. The moist mass was forced through a No. 8 hand screen and dried on trays at 100° F. for 16 hours. The material was then broken on a No. 16 hand screen.

FORMULATION III

Lactose U.S.P.	89.5%
Tragacanth U.S.P.	2.0%
Cornstarch	5.0%
Talcum U.S.P.	3.0%
Magnesium stearate U.S.P.	0.5%
3A Alcohol 50%	*q.s.*

The lactose and tragacanth were mixed and then moistened with the alcohol solution. The moist mass was screened through a 10-mesh sieve and dried with circulating dehumidified air for 16 hours. The dried material was then broken on a No. 16 hand screen and mixed with the cornstarch, talcum, and magnesium stearate.

The fourth formula is a simple mixture of the ingredients which are

FORMULATION IV

Lactose U.S.P.	94.5%
Cornstarch	5.0%
Magnesium stearate U.S.P.	0.5%

These materials were passed through a 20-mesh screen and then mixed.

Granulations were prepared in batches of 20,000 tablets, ten batches with conventionally-processed lactose, ten batches with spray-dried lactose. They were manufactured in a model A-200 Hobart planetary mixer. For each series, the mixing time, mixing speed, and amount of granulating liquid were the same. The granulations were compressed into 150-mg. tablets on a single rotary, 16-station press set to operate at 27,000 tablets per hour. The punches were $^{9}/_{32}$ in. diameter standard concave, uppers bisected and lowers monogrammed. Plain steel dies were used.

The results of the various tests to be described are the average of ten determinations.

Test Methods

Moisture Content.—Moisture content of the granulations was determined on a Cenco moisture balance operating at 120 v. with a 125-w. infrared lamp. The test was continued until the granulation changed color or until three consecutive readings at 1-min. intervals were the same.

Hardness.—Hardness was read on the hardness tester manufactured by the Strong-Cobb Arner Company. This unit was modified to operate from a compressed air line.

Friability.—Friability was measured with a Roche Friabilitor using a 4-min. cycle.

Disintegration Time.—Disintegration times were taken on the U.S.P. apparatus; no disks or holders were used.

Particle Size Analysis.—Sieve sizing was done on a Ro-Tap testing sieve shaker using stainless steel U.S. Standard sieve series in 20, 30, 40, 50, 60, and 80 mesh sizes and operating for 15 minutes. Each sample tested weighed 100 Gm.

Angle of Repose.—Angle of repose was measured by the cone method using a cathetometer and a 4-in. diameter tripod.

Flow Rate.—Flow rate was determined by timing the passage of 250 Gm. of granulation through a stainless steel funnel with a 1-in. orifice.

TABLE I.—INFLUENCE OF STORAGE ON DISINTEGRATION TIMES, IN MINUTES[a]

	R.T.		40° C.		50° C.		60° C.	
	A	B	A	B	A	B	A	B
Initial	12′25″	11′05″	...	...	...	...	...	...
4 Weeks	10′50″	10′40″	11′10″	10′50″	12′00″	10′40″	9′00″	9′25″
8 Weeks	11′15″	10′10″	11′05″	9′45″	12′40″	9′55″	6′10″	7′55″
12 Weeks	11′10″	9′30″	10′50″	9′25″	12′30″	9′35″	4′45″	7′20″

[a] A, conventionally-processed lactose; B, spray-dried lactose.

TABLE II.—INFLUENCE OF STORAGE ON HARDNESS, KG./SQ. IN.[a]

	R.T.		40° C.		50° C.		60° C.	
	A	B	A	B	A	B	A	B
Initial	5.9	8.5	...	...	...	...	...	...
4 Weeks	6.2	8.9	6.2	7.5	5.9	7.7	5.5	7.7
8 Weeks	6.1	8.4	6.2	8.0	6.1	7.6	4.5	7.6
12 Weeks	5.9	8.4	6.3	8.4	5.8	7.9	4.1	7.0

[a] A, conventionally-processed lactose; B, spray-dried lactose.

TABLE III.—INFLUENCE OF STORAGE ON FRIABILITY, % LOSS[a]

	R.T.		40° C.		50° C.		60° C.	
	A	B	A	B	A	B	A	B
Initial	0.34	0.20	...	...	...	...	...	...
4 Weeks	0.28	0.17	0.33	0.23	0.37	0.28	0.44	0.23
8 Weeks	0.28	0.18	0.34	0.20	0.37	0.21	0.36	0.21
12 Weeks	0.34	0.16	0.39	0.21	0.39	0.22	0.34	0.20

[a] A conventionally-processed lactose; B, spray-dried lactose.

RESULTS AND DISCUSSION

Formulation I.—As is evident from the data in Tables I, II, and III, the tablets were physically satisfactory. At the end of the 12-week test period, disintegration times were lower than initially at all temperatures. The tablets of spray-dried lactose disintegrated a little more rapidly except at 60°. Times for both types of tablets were well within U.S.P. limits.

Both sets of tablets exhibited good hardnesses with the conventional lactose always associated with the lower results. With time and temperature, there were changes downward from the initial figures but all the tablets were satisfactory except those of conventional lactose at 60°.

In friability the tablets containing spray-dried lactose suffered losses approximately one-half those of the conventional lactose regardless of time or temperature. The results for both sets of tablets remained quite constant under all storage conditions. It may be noted from the tables that even for those tablets which had a relatively poor hardness, the friability was not adversely affected.

A visible color change appeared in the spray-dried lactose tablets at 50° and 60°, a change confirmed by reflectance readings. However, the set of tablets from conventionally-processed lactose retained its original whiteness. The flow rates of the two granulations were very much alike, a fact confirmed by the similarities in the angles of repose. Spray-dried lactose gave slightly better results. On sieve analysis, both granulations contained mostly fine material, 78% of the conventional type and 89% of the spray-dried passing through a No. 60 sieve. Moisture content ranged between 3.3% and 5.3% but a correlation between moisture content and other physical characteristics was not apparent.

Formulation II.—Table IV shows that disintegration times were very rapid at room temperature throughout the test period. They rose gradually with storage at 40° and 50° with a severe increase at 60°. At this temperature, the tablets of spray-dried lactose disintegrated more rapidly than those of conventionally-processed lactose. Melting of the stearic acid or some change in the starch may have produced this effect.

Both types of tablets behaved similarly in the hardness test as evidenced by the data in Table V. At room temperature, there was a progressive softening of the tablets; this did not occur at the other temperatures. The tablets of conventionally-processed lactose were generally harder than those of spray-dried lactose.

Friability results shown in Table VI indicated tnat the conventionally-processed lactose produced better tablets initially but the losses became almost identical in all the later tests except the one at 60° for 12 weeks. These losses represented a large increase over the initial levels.

In flow rate and angle of repose, the conventional lactose averaged slightly better than the spray-dried. Moisture content averaged 0.7% higher in the granulations made from conventionally-processed lactose. A comparison of individual batch results gave no apparent correlation of moisture with hardness, with friability, or with disintegration time.

On sieve analysis, the granulations from spray-dried lactose had almost equal amounts caught on all screens with the No. 20 having the least. On the other hand, the granulations from the conventionally-processed lactose had a 43% cut passing through the 80-mesh screen with the second largest (19%) caught on the No. 80. Nevertheless, tableting characteristics of the two were very similar.

TABLE IV.—INFLUENCE OF STORAGE ON DISINTEGRATION TIME, IN MINUTES[a]

	R.T.		40° C.		50° C.		60° C.	
	A	B	A	B	A	B	A	B
Initial	0′15″	0′40″	...	...	...	...	...	...
4 Weeks	0′15″	0′50″	0′20″	1′05″	0′40″	2′05″	8′15″	10′30″
8 Weeks	0′15″	0′55″	0′20″	1′20″	2′30″	5′30″	22′30″	19′00″
12 Weeks	0′15″	0′50″	0′25″	1′40″	4′10″	7′25″	28′15″	22′40″

[a] A, conventionally-processed lactose; B, spray-dried lactose.

TABLE V.—INFLUENCE OF STORAGE ON HARDNESS, KG./SQ. IN.[a]

	R.T.		40° C.		50° C.		60° C.	
	A	B	A	B	A	B	A	B
Initial	5.2	5.2	...	...	...	...	...	...
4 Weeks	5.1	5.1	5.1	5.1	5.2	5.2	5.5	5.3
8 Weeks	4.9	4.7	5.2	5.1	5.4	5.2	5.2	5.1
12 Weeks	4.5	4.4	4.9	5.1	5.1	5.2	5.4	5.1

[a] A, conventionally-processed lactose; B, spray-dried lactose.

TABLE VI.—INFLUENCE OF STORAGE ON FRIABILITY, % LOSS[a]

	R.T.		40° C.		50° C.		60° C.	
	A	B	A	B	A	B	A	B
Initial	0.18	0.32	...	...	...	...	...	...
4 Weeks	0.45	0.51	0.46	0.47	0.56	0.55	0.53	0.51
8 Weeks	0.49	0.48	0.46	0.51	0.56	0.56	0.57	0.59
12 Weeks	0.52	0.55	0.48	0.49	0.57	0.62	0.60	0.73

[a] A, conventionally-processed lactose; B, spray-dried lactose.

By reflectance measurement, the tablets of spray-dried lactose were darker even when freshly made and became more so at 60°, the change being 7.3% whereas the tablets of conventionally-processed lactose changed only 2%. The difference between the two initially was 8.4% and increased to 13.7%.

Formulation III.—From Table VII it may be seen that both sets of tablets were almost identical in disintegration time at each storage condition. There were decreases with time and with temperature. All test results were low.

On the average, the spray-dried lactose produced the superior hardnesses as is indicated by the data in Table VIII. These increased from the fourth to the twelfth week at all temperatures with the room temperature samples increasing about half a unit while the 40°, 50°, and 60° samples ended up almost equal with each other and slightly below the initial level. The conventionally-processed lactose tablets softened under all conditions with the room temperature samples falling one unit and the other temperature results dropping slightly less.

In the friability tests, the conventionally-processed lactose tablets were very poor, two of the batches capping in the initial test and continuing to do so under the various storage conditions as shown in Table IX. Most of the other batches capped also but in a random pattern. Only two batches did not cap at all. As a consequence at all times and conditions, average friabilities were 0.9% or worse. On the other hand, the spray-dried lactose gave very good results at room temperature and satisfactory results at 50°, but rather poor at 40° and at 60°.

Tablets of spray-dried lactose darkened slightly more than those of conventionally-processed lactose at the accelerated conditions. There were small differences in flow rate and angle of repose. Both granulations contained many fines, the spray-dried lactose having more—93.6% passing through a No. 80 screen as against 81.7% for the conventional.

Formulation IV.—It was impossible to make adequate tablets from conventionally-processed lactose even when it was in the form of coarse (60–80 mesh) free-flowing granules. The tablets would not hold together. Therefore, the following data refer only to spray-dried lactose.

Disintegration times decreased with time at room temperature and reached their lowest points after 4 weeks at the other three storage conditions. These

TABLE X.—INFLUENCE OF STORAGE ON DISINTEGRATION TIME, IN MINUTES

	R.T.	40° C.	50° C.	60° C.
Initial	7′50″	...	...	...
4 Weeks	7′45″	2′45″	1′45″	2′25″
8 Weeks	5′40″	2′50″	2′20″	4′50″
12 Weeks	4′30″	2′55″	3′30″	7′20″

TABLE XI.—INFLUENCE OF STORAGE ON HARDNESS, KG./SQ. IN.

	R.T.	40° C.	50° C.	60° C.
Initial	5.7	...	...	...
4 Weeks	6.4	6.0	6.5	6.3
8 Weeks	6.1	6.3	6.4	6.0
12 Weeks	5.9	6.1	6.3	5.9

TABLE XII.—INFLUENCE OF STORAGE ON FRIABILITY, % LOSS

	R.T.	40° C.	50° C.	60° C.
Initial	0.07	...	...	...
4 Weeks	0.18	0.27	0.25	0.29
8 Weeks	0.30	0.29	0.26	0.29
12 Weeks	0.34	0.30	0.26	0.29

TABLE VII.—INFLUENCE OF STORAGE ON DISINTEGRATION TIME, IN MINUTES[a]

	R.T.		40° C.		50° C.		60° C.	
	A	B	A	B	A	B	A	B
Initial	2′05″	2′05″	...	...	...	...	...	...
4 Weeks	1′50″	1′30″	1′20″	1′05″	0′50″	1′00″	0′30″	0′45″
8 Weeks	1′45″	1′20″	1′10″	0′55″	0′45″	0′55″	0′30″	0′35″
12 Weeks	1′40″	1′30″	1′05″	1′00″	0′45″	0′50″	0′30″	0′35″

[a] A, conventionally-processed lactose; B, spray-dried lactose.

TABLE VIII.—INFLUENCE OF STORAGE ON HARDNESS, KG./SQ. IN.[a]

	R.T.		40° C.		50° C.		60° C.	
	A	B	A	B	A	B	A	B
Initial	4.7	5.0	...	...	...	...	...	...
4 Weeks	4.3	4.9	4.2	4.6	4.0	4.4	4.1	4.6
8 Weeks	4.0	5.0	4.0	4.7	4.1	4.8	3.9	4.8
12 Weeks	3.7	5.4	3.9	4.8	3.8	4.9	3.8	4.9

[a] A, conventionally-processed lactose; B, spray-dried lactose.

TABLE IX.—INFLUENCE OF STORAGE ON FRIABILITY, % LOSS[a]

	R.T.		40° C.		50° C.		60° C.	
	A	B	A	B	A	B	A	B
Initial	0.96	0.16	...	...	...	...	...	...
4 Weeks	0.95	0.21	1.05	0.71	1.43	0.36	0.72	0.71
8 Weeks	0.91	0.20	1.31	0.76	1.33	0.32	1.49	0.44
12 Weeks	1.03	0.26	1.23	0.42	0.95	0.39	1.14	0.60

[a] A, conventionally-processed lactose; B, spray-dried lactose.

latter then rose during the next 8 weeks until the 60° samples approached the initial result at room temperature (Table X).

Table XI indicates that all hardness results were greater than the initial one. For room temperature, 50°, and 60° they reached a peak at 4 weeks and then declined. Although the hardness readings decreased at 50° and 60° during the last 8 weeks, the disintegration times increased.

Friability was remarkably low at the beginning of the study but it increased with time, the highest level being at room temperature (See Table XII). All results were satisfactory however. Color change was very strong, being 13.5% darker after 12 weeks at 60° than initially.

An inconvenience with spray-dried lactose is that on aging it darkens appreciably more than the conventionally-processed material. This fact has been indicated in the discussion of each formulation. Table XIII compares the reflectance values of the tablets at the beginning and the end of the study along with the values for samples of the two types of lactose.

TABLE XIII.—EFFECT OF STORAGE ON COLOR STABILITY OF LACTOSE AND LACTOSE TABLETS ACCORDING TO REFLECTANCE MEASUREMENTS

Formulation[a]	Zero Time	12 Weeks' Storage at— R.T.	40° C.	50° C.	60° C.
88.5% lactose					
I A	63.0	62.0	63.0	63.0	62.0
I B	62.0	62.0	59.7	54.3	50.3
94.5% lactose					
II A	67.0	67.0	66.0	66.0	65.0
II B	59.4	58.6	57.0	54.7	51.3
89.5% lactose					
III A	59.8	59.4	58.0	57.7	55.9
III B	57.8	57.8	57.0	53.3	51.9
94.5% lactose					
IV B	57.2	56.8	52.2	46.7	43.3
100% lactose					
A	99.0	99.0	89.0	91.0	89.0
B	88.0	88.0	62.0	63.0	38.0

[a] A, conventionally-processed lactose; B, spray-dried lactose.

Why spray-dried lactose discolors more than conventionally-processed lactose has not been specifically elucidated. However, it does contain larger amounts of impurities because the mother liquor is included in it. It is exposed to high heat while passing through the spray dryer but, theoretically, the evaporation of the moisture cools the crystals and prevents them from overheating. However, the other ingredients in the formulation afford some protection and indicate one method of attack on the problem.

A seeming paradox occurred between the data on flow and on weight control of the tablets (Table XIV).

The formulation with the slowest flow rate (Formulation III) produced the tablets with the most accurate weight control while the formulation with the most rapid flow (Formulation II) had the greatest weight variance.

Reference has been made several times to sieve sizing. The complete results are available in Table XV.

SUMMARY AND CONCLUSIONS

1. In three out of four formulations examined, tablets made from spray-dried lactose exhibited better physical qualities than those made from conventionally-processed lactose.

2. Spray-dried lactose is particularly useful because tablets can be made from it without wet granulating or slugging.

3. Spray-dried lactose darkens much more readily than conventionally-processed lactose.

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TABLE XIV.—FLOW PROPERTIES AND WEIGHT VARIATION

Tests	Formulation[a] I A	I B	II A	II B	III A	III B	IV B
Flow rate (seconds)	8.57	8.44	7.60	8.14	10.48	11.21	8.35
Angle of repose	36°29′	34°45′	36°10′	37°29′	36°55′	37°39′	36°08′
Mean weight (mg.)	151.16	150.33	148.84	148.78	148.47	150.34	149.36
Standard deviation (mg.)	±1.10	±1.14	±1.50	±1.72	±0.55	±0.58	±0.70

[a] A, conventionally-processed lactose; B, spray-dried lactose.

TABLE XV.—SIEVE ANALYSIS, %

% Found On	Formulation[a] I A	I B	II A	II B	III A	III B	IV B
20 mesh	1.08	2.06	1.76	5.82	0.12	0.33	...
30 mesh	4.23	4.16	5.91	14.78	0.81	1.13	...
40 mesh	3.88	1.95	7.10	14.24	0.67	0.55	...
50 mesh	7.01	1.63	13.72	19.48	2.32	0.95	...
60 mesh	5.50	0.90	9.30	11.78	2.36	0.72	...
80 mesh	19.17	4.16	19.25	17.57	12.04	2.71	...
Through 80 mesh	59.13	85.13	42.96	16.33	81.68	93.61	100.00

[a] A, conventionally-processed lactose; B, spray-dried lactose.

Stability of Antibacterial Preservatives in Parenteral Solutions II

Microbiological Turbidimetric Assay Method for Preservative Content

By P. C. EISMAN, E. EBERSOLD, J. WEERTS, and L. LACHMAN

An accurate and rapid microbiological method for assaying chemical preservatives of nonantibiotic nature has been developed which employs a time-tested, proven procedure. Preservative concentration is measured by a turbidimetric procedure using *Escherichia coli* as the test organism. The method has been applied to chlorobutanol, phenol, and -chloro-beta-phenylethyl alcohol and compares favorably with chemical analytical methods.

IN CARRYING out stability studies on chemical preservatives in pharmaceutical solutions, it is essential that the methods employed accurately establish the preservative content. Analytical methods invariably have involved chemical procedures of varying degrees of complexity. Although regarded as possessing reliability, such assays may require chemical manipulation and equipment which are not always readily available. From a theoretical standpoint it is conceivable that while a chemical analysis may indicate appreciable loss of preservative content because of degradation, the breakdown products *per se* may possess intrinsic antibacterial activity.

In view of the generally accepted concept that preservative as well as antibiotic content is best expressed on the basis of antimicrobial activity, an attempt was made to develop an accurate and rapid turbidimetric assay similar to those generally employed for antibiotics. An extensive search of the literature has revealed no reports on the existence of a bioassay for chemical preservatives of nonantibiotic nature.

Initial attempts on our part (1) to arrive at a bioassay method involved the addition to the sample being assayed of a standardized number of *E. coli* organisms. At intervals, 1-ml. portions were removed, which were immediately plated for survivor count. Curves were then plotted of per cent survivors *vs.* time for each concentration of preservative. The time required to produce 50% killing was obtained from the plots. A standard curve was then drawn of the time required to destroy 50% of the organisms *vs.* the corresponding concentrations of preservative. Using the 50% killing time value of a sample of preservative solution under test and placing it on the standard curve, an estimate of the concentration of preservative was obtained. However, this procedure proved too cumbersome and time-consuming and, moreover, yielded poorly reproducible results.

As presently applied, the assay has proved to be rapid and reliable for the determination of chlorobutanol, phenol, and *p*-chlorophenylethyl alcohol, the only preservatives investigated thus far. With minor modifications, the procedure is similar to that of the turbidimetric assay employed in our laboratory for viomycin as described by Kersey (2).

In essence, the method consists of the addition graded concentrations of the preservative to a series of test tubes containing nutrient broth seeded with *E. coli*. After an incubation of approximately 3.5 hours the turbidity of each tube is measured for graded response by means of a photoelectric colorimeter fitted with a color filter. The potency of the sample is established by comparing the response of the test organism to the preservative under test with that observed in the standard reference series. Because of the rapidity of the test and the relatively heavy *E. coli* inoculum, strictly sterile techniques need not be necessarily observed.

EXPERIMENTAL

Culture and Medium.—Stock cultures of *E. coli*, strain Su 2, are maintained on BBL trypticase soy agar slants. Prior to the assay, the culture is transferred to two fresh agar slants which are incubated overnight at 37°. The growth from these slants are suspended in sufficient sterile 0.9 per cent sodium chloride solution to yield a light transmission reading of 10–12% (equivalent to an optical density of approximately 1.0 at a wavelength of 580 mμ in a Bausch and Lomb Spectronic 20 colorimeter). One milliliter of this suspension is added to 100-ml.

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TABLE I.—REPRODUCIBILITY OF ASSAYS PERFORMED ON DIFFERENT DAYS

Assay Method	Assay No.	*p*-Chloro-beta-phenylethyl Alc., % Found	Chlorobutanol, % Found	Phenol, % Found
Microbiological	1	0.121	0.255	0.590
	2	0.126	0.270	0.565
	3	0.116	0.249	0.565
	4	0.108	0.262	0.535
	5	0.111	0.256	0.543
	6	0.129	0.238	0.525
	7	0.120	0.262	0.543
	8	0.124	0.250	0.543
	9	0.117	0.262	0.535
	10	0.119	0.250	0.550
	11	0.130	0.245	...
	12	0.131	0.252	...
	13	0.120	0.253	...
	14	0.124	0.270	...
	15	...	0.262	...
Means ± standard errors		0.121 ± 0.0017	0.255 ± 0.0022	0.549 ± 0.0060
95% Confidence limits		0.1173 – 0.1247	0.2503 – 0.2590	0.5354 – 0.5626
Chemical		0.117	0.250	0.560

TABLE II.—ACCURACY OF ASSAYS CALCULATED FROM DIFFERENT POINTS OF STANDARD SLOPE[a]

Preservative	Assay Method	Preservative Content, % in Sample No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
Chlorobutanol	Chemical	0.47	0.44	0.34	0.25	0.14	0.04
	Microbiol.	0.46	0.43	0.35	0.22	0.14	0.04
Phenol	Chemical	1.02	0.62	0.27	...	...	...
	Microbiol.	1.05	0.61	0.24	...	...	...
p-Chloro-beta-phenylethyl alcohol	Chemical	0.22	0.19	0.16	0.13	...	...
	Microbiol.	0.23	0.20	0.17	0.13	...	...

[a] All values based upon microbiological assay values for each different preservative were obtained from a single standard slope.

amounts of BBL trypticase soy broth. This inoculated broth is now ready for use in the assay.

Standard Solutions.—The following standard reference solutions are prepared in pH 4.0 (0.275 *M*) citric acid–sodium phosphate buffer: chlorobutanol, 0.5, 0.4, 0.3, 0.2, 0.1, and 0.05%; phenol, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, and 0.2%; and *p*-chloro-beta-phenylethyl alcohol, 0.262, 0.225, 0.187, 0.15, 0.112, and 0.075%.

Samples.—All samples for assay are diluted to contain not more than the maximum concentration of preservative employed in the standard reference series.

Assay Procedure: One-milliliter amounts of each standard reference concentration and of each unknown solution are added to test tubes in quadruplicate. As controls, 1.0 ml. of buffer solution containing no preservative is included in each assay. Nine milliliters of inoculated broth is then added to each tube. A syringe pipetting assembly (Cornwall type) fitted with a two-inch cannula is used for the rapid dispensing of the broth. Each tube is thoroughly shaken and placed in a water bath uniformly maintained at 37° until the control tubes have an optical density reading of approximately 0.35 (usually 3.5 hours of incubation). Further growth of the test organism is immediately stopped by the addition of 0.5-ml. amounts of formalin, diluted 1:3, employing the syringe pipet for this purpose.[1] The optical density of each tube of broth is obtained with the colorimeter at a wavelength of 530 mμ.

[1] The use of buffers with pH values greater than 4.0 will result in short periods of incubation.

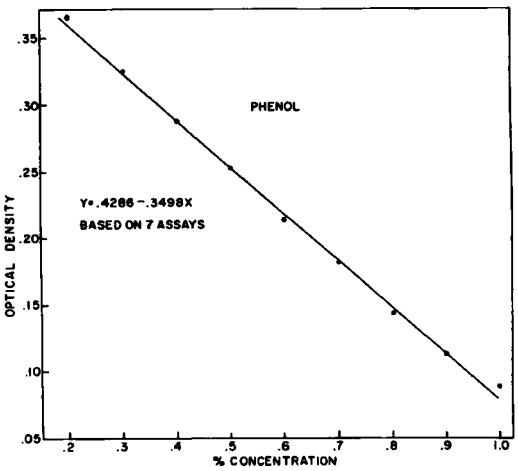

Fig. 1.—Computed line based on the values obtained from 7 assays.

The colorimeter is adjusted with clear, sterile broth to a reading of *O* optical density (*i.e.*, 100% light transmission). For each turbidity reading, the contents of the tube are transferred to a 3/4 in. × 6 in. test tube made expressly for the Spectronic 20 colorimeter by Bausch and Lomb.[2] After each reading, the broth is removed by suction. The values obtained for each set of quadrupli-

[2] Catalog No. 33-29-37.

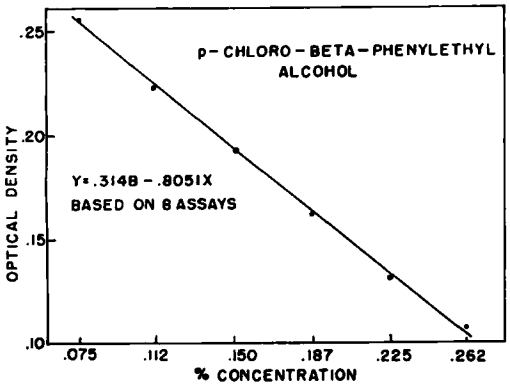

Fig. 2.—Computed line based on the values obtained from 8 assays.

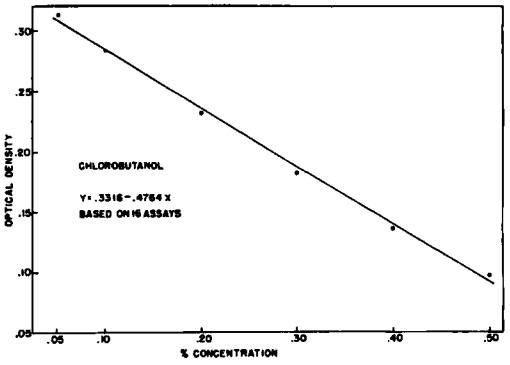

Fig. 3.—Computed line based on the values obtained from 16 assays.

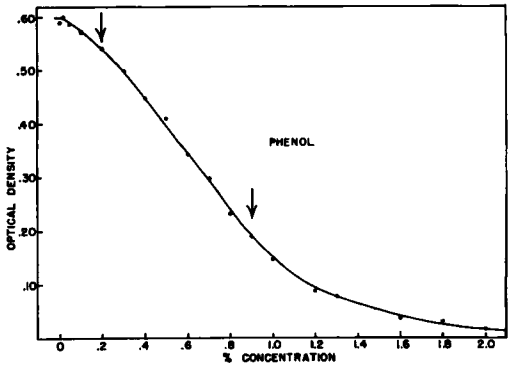

Fig. 4.—A typical sigmoid curve obtained when % concentration is plotted against optical density. The zone of straight line relationship is indicated by the arrows.

cate tubes are averaged. The average optical density values obtained for the standard reference concentrations are plotted on arithmetic paper against the corresponding preservative concentrations, and a straight line is drawn. Potencies of samples being assayed are then determined from the standard slope. Figures 1, 2, and 3 represent computed lines for phenol, *p*-chloro-beta-phenylethyl alcohol, and chlorobutanol based on 7, 8, and 16 assays, respectively, each assay being performed on different days. A straight line relationship was proved by the analysis of variance. Figure 4 shows the typical sigmoid curve for phenol. For our potency estimations only the linear zone, as indicated by the arrows, was used.

RESULTS

Since the relationship between the variables (*i.e.*, the per cent concentration of preservative and the corresponding observed optical density) is linear, we have compared the standard dose-response line as drawn by inspection with one drawn by the method of least squares. In almost all instances these two drawn lines, with respect to each of the three preservatives studied, were virtually superimposable.

As indicated in Table I, there is excellent agreement between the values derived for the three preservatives by chemical analysis and those obtained by the turbidimetric bioassay with *E. coli*.

That assay values of unknown samples calculated from different portions of the standard reference slope agree with corresponding values obtained by chemical analysis is shown in Table II.

SUMMARY

1. An accurate method is described for the turbidimetric assay of chlorobutanol, phenol, and *p*-chloro-beta-phenylethyl alcohol, employing *E. coli* as the test organism and requiring a $3^1/_2$-hour incubation period. The method is essentially the same as that currently used for viomycin and other antibiotics.

2. The procedure for establishing standard reference slopes for these preservatives is described.

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Evaluation of Antispasmodic Activity in the Intact Dog

By STUART A. TURKANIS and HOWARD J. JENKINS

Results of a method developed to determine the comparative effectiveness of the active components of an antispasmodic formula and their combination in the surgically intact animal are presented. These confirm established relative potencies of the compounds involved and indicate that the combination of the formula under investigation represents a potentiating union.

THE QUALITATIVE and quantitative effects of atropine and chemically related cholinergic blocking agents have been demonstrated by numerous investigators using both *in vivo* and *in vitro* pharmacologic procedures. In most instances, some sort of surgical intervention of the gastrointestinal tract has been involved in the *in vivo* studies to date. Excellent *in vitro* biological assays such as those reported by Luduena and Lands are available (1). Using ileal strips, these investigators have achieved procedures of general acceptance in the pharmacologic screening of cholinergic blocking agents.

One of the problems confronting *in vivo* methods has been that involved with the tachyphylactic response to the more potent cholinergic blocking agents, evidenced in the progressive depression of gastrointestinal sensitivity and described by Seevers, *et al.* (2), in 1954 and by Quigley, *et al.* (3), in 1937. Since the reliability of a biological assay is known to be dependent upon the stability of the dose-response relationship, the tachyphylactic response here encountered constitutes a barrier to the successful development of a biological assay method for the aforementioned agents.

The quantitative determination of the cholinolytic activity of atropine and its belladonna relatives individually and in combination *in vivo* constitutes the basis of this investigation.

EXPERIMENTAL

Pressure changes occurring in the sensing element, a rubber sheath, in response to gastrointestinal movements were transmitted by means of its partially encased Cantor tube (D-111, 12, French) to a sensitive Sanborn transducer where these changes were converted to electrical impulses. The electrical impulses were then, after amplification, conveyed to a Sanborn two-channel recorder.

Along with measurement of gastrointestinal tract activity of the test animal, the respiratory activity was recorded concomitantly by means of a pneumograph, a second Sanborn transducer and Sanborn strain gauge amplifier being employed for this recording.

The test animals were mongrel dogs of either sex ranging in weight from 11.85 to 18.0 Kg. During the study they were fed once daily and supplied with water *ad libitum*. Each dog was anesthetized before each test with 30 mg./Kg. of pentobarbital sodium administered intravenously. The level of anesthesia that was achieved was just insufficient to abolish the swallowing reflex. No dog was used more often than every 48 hours for the testing of these compounds.

In all the tests, the Cantor tube reached approximately the same level in the gastrointestinal tract. At the completion of this series of tests a dog was sacrificed to reveal that the rubber sheath was located in the pyloric portion of the stomach.

All drugs were administered intravenously by means of an in-dwelling 18-gauge hypodermic needle, which was attached by a rubber tube to a 50-ml. buret containing 0.9% saline solution.

In the series of tests involving the response of the gastrointestinal tract to methacholine chloride, tachyphylaxis was not observed.

In view of the possibility of achieving a complete block with atropine sulfate and the chemically related cholinergic blocking agents of belladonna, the range values of the blocking dose of atropine sulfate were determined experimentally prior to the study to be 0.067 to 0.10 mg./Kg. Ten minutes after the intravenous administration of 0.10 mg./Kg. of atropine sulfate, no increase in gastrointestinal activity was achieved with an indefinitely large dose of methacholine chloride, although profuse salivation was evidenced.

In preliminary experiments, it was noted that the greater the dose of the spasmogen, methacholine chloride, the greater the response and the greater the duration of the response. Termination of the response was assumed when the base line of the recording returned approximately to its predose level. The mean average duration of the response to methacholine chloride, according to the above criterion, was 8 minutes, with a range of 4–10 minutes. To allow a margin of safety, the spasmogen was administered at 15-minute intervals throughout the entire series of the tests. Thus, cumulative effects of methacholine chloride were avoided.

The onset of atropine and hyoscyamine activity, which was indicated by a fall in the base line of the graph, was shown to occur within 5 minutes of the

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intravenous administration of these cholinergic blocking agents. The establishment of the atropine or the hyoscyamine partial cholinergic block of the gastrointestinal tract was demonstrated in every instance within 10 minutes following the intravenous administration of these agents. The onset of hyoscine activity was more variable than that of atropine or hyoscyamine, and the establishment of the partial cholinergic block of hyoscine required at least 30 minutes following the intravenous administration of the drug.

From the preliminary studies, it became evident that the antispasmodic activity of atropine and related belladonna alkaloids is sufficiently enduring that cumulative effects would almost certainly occur if several doses of antispasmodic were administered during the course of one determination. This would, of course, make difficult or impossible the evaluation of the effectiveness of a single dose of antispasmodic. Consequently, the individual alkaloids and their combinations were given in this method only one dose per determination.

Methacholine chloride, the spasmogen, was administered in varying doses with the intent of obtaining a methacholine chloride$_{50}$, or Me$_{50}$, a dose of methacholine chloride the response to which is reduced by the antispasmodic to 50% of that to a preantispasmodic dose of this drug. The extent of the inhibition of methacholine chloride was determined and expressed as per cent of the preantispasmodic response.

A minimum of three doses was employed in order to obtain a Me$_{50}$: a high dose with an inhibition of less than 50%, a low dose with an inhibition of more than 50%, and a middle dose with an inhibition of approximately 50%. Each postantispasmodic dose of methacholine chloride in mcg./Kg. was plotted against the corresponding per cent reduction of the preantispasmodic response to methacholine chloride on logarithmic probability paper. The line to fit these data was drawn by inspection and the Chi^2 test was applied to determine the goodness of fit. From this line of the plotted data the Me$_{50}$ was read directly.

Throughout a particular determination, only one preantispasmodic dose of methacholine chloride was used, and the per cent reduction of the postantispasmodic methacholine responses was determined from this value.

The Me$_{50}$ of atropine sulfate at 0.030 mg./Kg. was calculated to be 9.25 mcg./Kg. To assess a relative activity with respect to the other antispasmodics and a combination of belladonna alkaloids[1] (CBA) the Me$_{50}$ 9.25 of each of these agents was determined. A minimum of three doses of antispasmodic was employed in order to obtain an antispasmodic dose with an Me$_{50}$ 9.25: a dose with an Me$_{50}$ above 9.25, a dose with an Me$_{50}$ below 9.25, and a dose with an Me$_{50}$ between these values. The log of each Me$_{50}$ was plotted against the log of the corresponding dose of the antispasmodic. From the resulting linear relationship, the Me$_{50}$ 9.25 of that particular antispasmodic or combination of antispasmodics was read directly from the graph and was calculated from the regression equation.

Each antispasmodic was assigned a relative potency value which was based on the reciprocal of its Me$_{50}$ 9.25 value multiplied by the Me$_{50}$ 9.25 of atropine sulfate, 0.030 mg./Kg. Thus atropine was given a value of unity and the other agents, including CBA, were listed according to their relative potencies as multiples of this number.

The individual Me$_{50}$ 9.25 doses of the three active components were weighted according to the relative concentrations of each in the combination, CBA. The sum of these weighted means was then divided by the sum of the relative concentrations of the components and the quotient obtained was considered to represent the calculated Me$_{50}$ 9.25 of CBA. The actual or the experimental Me$_{50}$ 9.25 value of CBA was obtained as were the component values. Comparison of the calculated and the experimental values for CBA effectiveness indicates that the combination constitutes an activity greater than the summed activity of the components.

DISCUSSION

This investigation has produced a method for evaluating the antispasmodic effectiveness of anti-

TABLE I.—THE ANTISPASMODIC ACTIVITY OF BELLADONNA ALKALOIDS

Alkaloid	Dose, mg./Kg.	Me$_{50}$, mcg./Kg.	A[a]	B[b]
Atropine sulfate	0.030	9.25	4.22	12.8
			8.44	55.3
			16.88	70.2
			25.32	88.94
Hyoscyamine sulfate	0.010	4.7	3.13	14.7
			6.25	68.8
			9.38	90.0
Hyoscyamine sulfate	0.0125	6.8	3.67	7.5
			5.51	22.5
			7.34	65.0
Hyoscyamine sulfate	0.0150	14.25	4.41	15.6
			8.82	42.6
			17.6	53.0
			26.46	64.8
Hyoscine hydrobromide	0.005	7.9	2.5	10.0
			5.0	20.2
			10.0	59.4
Hyoscine hydrobromide	0.005	9.2	3.75	6.0
			6.25	44.0
			12.5	78.0
Hyoscine hydrobromide	0.007	6.2	3.68	36.7
			5.51	43.3
			11.0	73.3
Hyoscine hydrobromide	0.007	6.2	3.13	15.0
			6.25	57.5
			12.5	86.4
Hyoscine hydrobromide	0.0073	5.6	2.8	7.8
			5.6	44.6
			8.2	79.2
Hyoscine hydrobromide	0.009	7.9	4.72	2.9
			9.45	17.0
			15.7	33.3
			23.9	57.0
CBA	0.009	4.5	3.67	35.5
			5.5	61.9
			7.35	85.75
CBA	0.012	14.2	7.35	25.5
			14.7	50.0
			18.5	61.4
			22.1	68.2
CBA	0.015	19.5	5.0	10.0
			10.0	19.5
			16.65	59.7

[1] Kindly supplied as Donna by A. H. Robins and Co., Inc., Richmond, Va.

[a] A = Methacholine chloride mcg./Kg. [b] B = Per cent reduction.

TABLE II.—COMPUTATION OF CALCULATED VALUE OF THE Me_{50} 9.25 MCG./KG. FOR DONNA (CBA)

Alkaloid	Concentration in Donna	Actual Me_{50} 9.25 mcg./Kg.
Hyoscyamine sulfate	1037 × 0.01316	= 13.6469
Atropine sulfate	194 × 0.03000	= 5.8200
Hyoscine hydrobromide	65 × 0.00685	= 0.4453
	1296	19.9122[a]

[a] $\frac{19.9122}{1296}$ = 0.01536 mg./Kg. of Donna is the calculated value of the Me_{50} 9.25 mcg./Kg.

TABLE III.—ANTISPASMODIC ACTIVITY IN COMPARISON WITH ATROPINE SULFATE

Drug	Me_{50} 9.25 mcg./Kg.	Potency Compared to Atropine Sulfate
Atropine sulfate	0.0300	1.0
Hyoscyamine sulfate	0.01316	2.28
Hyoscine hydrobromide	0.00685	4.38
CBA	0.01120	2.68

cholinergic agents generally. The potency of such agents may be assessed in the procedure on the basis of comparison with an established standard. The advantage claimed for the method is that it is carried out in the intact dog without the necessity for surgical intervention in the placement of the sensing element. Thus, the same group of animals can be used repeatedly and without lengthy preparation in the determination involved.

The method developed has made possible a comparison of the relative effectiveness of the individual components of the combination CBA with the relative effectiveness of the combination. The results obtained have indicated potentiation of action in the combination.

Although atropine, hyoscyamine, and hyoscine were administered intravenously, it is very likely that similar results would be produced by oral administration, since these alkaloids are well absorbed from the gastrointestinal tract. Synthetic blockers should be studied, to be sure, by several routes of administration, since an agent which shows considerable cholinergic blocking activity parenterally could fail to achieve clinical usefulness because of poor absorption from the gastrointestinal tract.

Another possibility for further application of the method involves the direction of the alteration of the concentrations of the various alkaloids in such combinations as CBA in order to achieve an optimum combination of the component alkaloids, *i.e.*, one providing the greatest potentiation of the therapeutic benefits of the components consistent with the lowest incidence and least severity of untoward side effects.

SUMMARY

A biological assay employing the surgically intact dog is used to determine the relative activity of atropine sulfate, hyoscyamine sulfate, and hyoscine hydrobromide both alone and in combination on the gastrointestinal tract.

Indication of potentiation in the antispasmodic activity of CBA is provided by a comparison of the experimentally determined activity with the activity calculated from the activities of the components.

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(3) Quigley, J. P., *Proc. Soc. Expll. Biol. Med.*, **36**, 450 (1937).

ERRATUM

In the paper titled "Effects of Ionizing Radiation on Two Gelatin Fractions I. Material Preparation, Dosimetry, and Acid-Base Behavior" (1), the caption for Fig. 3 should be revised to read: ". . . after 5% dispersions of each were subjected to an irradiation dose of 2.0 Mrads."

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Solubilization of Salicylic Acid by Polysorbate 80 as Determined by Solubility Titration

By NATHAN A. HALL

A solubility titration has been used to study the solubilization of salicylic acid in water by polysorbate 80 since a liquid phase separates from supersaturated solutions to give a distinct turbidity end point. The ratio of solubilizate to solubilizer necessary for complete water-miscibility was also determined. The method should be of general applicability in studying phenols solubilized by polyoxyethylene surface-active agents. The effect on the system of including a fixed weight fraction of certain additives in the polysorbate 80 was investigated. The additives included a series of monohydroxy alcohols of decreasing dielectric constants, a group of polyhydroxy alcohols, polyethylene glycol 400, and Tween 20. Added monohydroxy alcohols increased or decreased the solubilizing power of polysorbate 80 in order of their polarity. Added polyhydroxy alcohols and polyethylene glycol 400 showed little effect. Added Tween 20 decreased the solubilizing power of polysorbate 80 in a linear fashion.

In the preparation of solubilized systems the formulator is faced with the problem of selecting the proper amount of solubilizing substance to prepare stable isotropic liquids for a specific purpose. Studies on micellular solubilization conducted near the critical micelle concentration of the surface-active agent are valuable in elucidating mechanisms of solubilization, but because they deal with low concentrations of both solubilizer and solubilizate they are of limited utility in designing a suitable formula where relatively large amounts of each may be required.

For a rapid means of determining the amount of solubilizer needed for a given solubilizate, titration with water has been recommended as a simple procedure (1–3). Results can be tabulated or plotted as a phase diagram from which the relative quantities of solubilizer and solubilizate can be determined for each situation. Titrations where the solubilizate is a liquid, such as a volatile oil, give good end points easily detected by formation of an opaque emulsion. The end point is not always readily discernible if the solubilizate is a solid, however. Guttman and Higuchi (4) have reported complex formation between a number of phenols and macromolecules including polyethylene glycols. Complexes separated as oily liquids. Mulley and Metcalf (5) studied the complex form between chloroxylenol and cetomacrogol, a surface-active polyethylene glycol ether, and observed a similar separation of an oily liquid. These observations suggested that a solubility titration procedure could be used to examine solubilization in water of the salicylic acid complex by polysorbate 80, and preliminary experiments showed the system to give a suitable turbidity end point. Practically, the solubilization of the polyethylene glycol derivative-salicylic acid complex reflects the solubilization of salicylic acid by polysorbate 80. Pharmaceutically the use of polysorbate 80 could be criticized since its ester linkage is susceptible to hydrolysis in aqueous salicylic acid solutions, but it served to illustrate the general applicability of the method and presented no problems in the short term experiments of this study.

This investigation revealed that there was a critical ratio of solubilizate to surface-active agent which gave maximum complete solubilization, namely, a ratio which was miscible with water in all proportions. Coles (6) has shown that glycerol could be used to lower the amount of polysorbate 80 needed to produce a water-miscible solution of vitamin A palmitate, and has studied the glycerol:polysorbate 80:vitamin A:-water system in some detail (8). It was decided, therefore, to examine the effect of some added alcohols and Tween 20 upon critical miscibility ratios of salicylic acid and polysorbate 80.

EXPERIMENTAL

Materials.—Commercial samples of polysorbate 80 and Tween 20 (Honeywill-Atlas Ltd., London, England) were used without purification. The same samples were used in all experiments. Salicylic acid B.P. was recrystallized from 70% ethanol, dried under vacuum, and reduced to a fine powder in an agate mortar. All monohydric alcohols were dehydrated, redistilled, and stored under anhydrous conditions. Ethylene glycol, propylene glycol, and glycerin were dehydrated by heating to 180°, cooled and stored in a desiccator charged with sulfuric acid. Polyethylene glycol 400 U.S.P. was used as supplied by Government Medical Stores, Singapore. Mixtures of polysorbate 80 and added substances were prepared by weight. Dehydrated glycerin was not miscible with polysorbate 80; there-

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fore the mixture was shaken vigorously to assure homogeneity just prior to withdrawing the sample.

Solubility Titration.—The method was adapted from that of O'Malley, *et al.* (1). Five-gram samples of the solubilizer were weighed into 50-ml. beakers containing a weighed quantity of salicylic acid and a magnetic stirring bar. Beakers were placed in a shallow water bath on the platform of a magnetic stirrer and stirred until complete solution resulted. The titration was carried out in an air-conditioned room and the water bath was maintained at 25 ±1° by the application of heat at irregular intervals. A strong light was used to illuminate the beaker during the addition of purified water from a buret mounted so that the tip was just above the surface of the stirring liquid. Turbidity which remained for 2 minutes was taken as the end point. Titrations were accomplished with polysorbate 80 alone and containing various additives listed in Table I.

At the turbidity end point the ratio of salicylic acid to polysorbate 80 and the per cent water by weight was calculated for each sample. The ratio was based upon only the polysorbate 80 present without the additive; thus it expressed the weight of salicylic acid solubilized in the presence of 1 Gm. of polysorbate 80 in the system studied. The solubility of salicylic acid at the concentrations examined in the system excluding the surface-active agent was not large enough to significantly affect the results. Figure 1 shows several titration curves for polysorbate 80 alone and containing 20% by weight of various alcohols. The 20% concentration was selected as being a convenient concentration showing a well-defined behavior by preliminary experiments.

Determination of the Critical Miscibility Ratio (CMR).—The ratio (salicylic acid:polysorbate 80) at which complete miscibility with water was observed was termed the "Critical Miscibility Ratio" (*CMR*). Ratios of salicylic acid to polysorbate 80 above the *CMR* would not form an isotropic system with all proportions of water and any ratio below the *CMR* would. Systems containing low concentrations of salicylic acid where its solubility in the dispersion medium significantly affects the ratio must be excluded from the definition. For salicylic acid the *CMR* was found applicable to solutions containing 5% or more of polysorbate 80 (*ca.* 0.75% salicylic acid). An indication of the probable *CMR* was obtained from the titration curve.

A more exact determination of the *CMR* was made by adding to the weighed solubilizing mixture known weights of salicylic acid calculated to give the desired ratio to three figures. Each sample was quantitatively transferred to 50-ml. ground glass-

TABLE I.—EFFECT OF VARIOUS ALCOHOLS ON THE CRITICAL MISCIBILITY RATIO (*CMR*) OF SALICYLIC ACID AND POLYSORBATE 80

Polysorbate 80 Containing	*CMR*, Salicylic Acid/ Polysorbate 80
No Additive	0.150
Methanol [33][a], 20%	0.150
Ethanol [24], 20%	0.150
iso-Propanol [20], 20%	0.145
n-Propanol [18], 20%	0.145
n-Butanol [17], 20%	0.140
Cyclohexanol [15], 20%	0.110
n-Pentanol [14], 20%	0.095
n-Hexanol [13], 20%	0.075
Ethylene glycol, 20%	0.150
Propylene glycol, 20%	0.150
Glycerol, 20%	0.150
Polyethylene glycol 400, 20%	0.150

[a] Approximate dielectric constants for monohydroxy alcohols (7) are given in brackets.

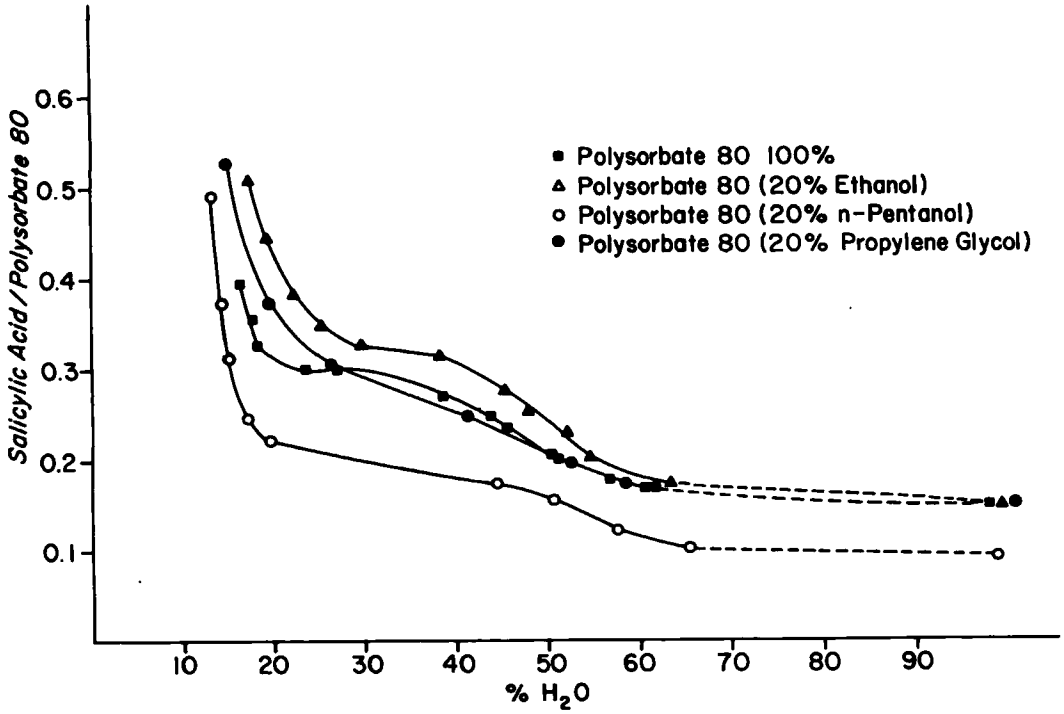

Fig. 1.—Titration curve for the system polysorbate 80 (with and without additives):salicylic acid:water.

TABLE II.—EFFECT OF TWEEN 20 ON THE CRITICAL MISCIBILITY RATIO (*CMR*) OF SALICYLIC ACID AND POLYSORBATE 80

Polysorbate 80 Containing	*CMR*, Salicylic Acid/ Polysorbate 80
No Additive	0.150
Tween 20, 20%	0.145
Tween 20, 40%	0.140
Tween 20, 60%	0.135
Tween 20, 80%	0.130
Tween 20, 100%	(0.130)

stoppered tubes with water and mixed. Each tube contained at least 90% water. The tubes containing the samples were suspended in a thermostatically controlled water bath at 25 ±0.1° for 24 hours. The tubes were then removed and turbidity was readily observable under a strong light. The highest ratio which showed no evidence of instability was taken as the *CMR*. Determinations were made in duplicate. Table I shows the effect on the *CMR* of various additives to polysorbate 80 and Table II shows the effect of mixtures of polysorbate 80 and Tween 20 on the *CMR*.

DISCUSSION

The titration curves of Fig. 1 are essentially phase diagrams. For systems containing percentages of water indicated, ratios of salicylic acid to polysorbate 80 above the line will not produce clear solutions; those below will. The titration curves can be used to calculate the amount of salicylic acid solubilized by a given weight of polysorbate 80 at known water concentrations and the reciprocal of the ratio can be used to calculate the amount of polysorbate 80 needed for a given weight of salicylic acid. The data may also be plotted as a three-component phase diagram if desired; however, as plotted for Fig. 1 the data are especially valuable to reflect comparative effects of added substances on solubilizing ability of polysorbate 80 for salicylic acid. Thus it is seen that ethanol assists considerably in the solubilization below 50% water but at high water concentrations it has little effect; propylene glycol has some power to increase solubilization at very low water concentrations but generally neither promotes nor inhibits the solubilization; *n*-pentanol generally depresses the solubilization.

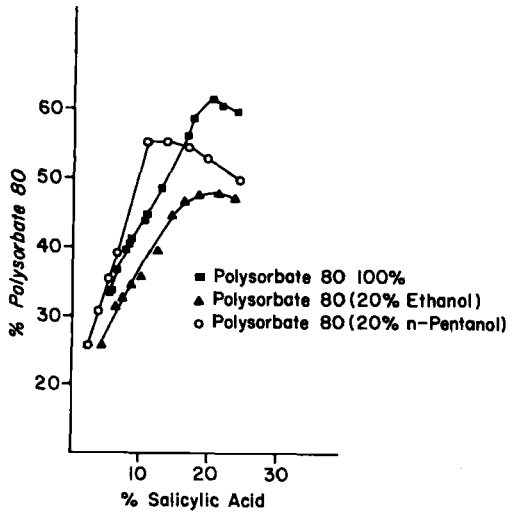

Fig. 2.—Titration data showing salicylic acid concentration at the turbidity end point as a function of polysorbate 80 concentration.

Another plot of the titration data in which the composition at the turbidity end point is used to determine the percentage of salicylic acid in the system as a function of the percentage of Tween 80 (all % by weight) is shown in Fig. 2. This shows in another fashion the solubilizing activity of polysorbate 80 and the maximum amount of salicylic acid which can be solubilized. The solubility of salicylic acid in the additives at the concentrations used does not significantly affect the plot. The solubilizing power of polysorbate 80 appears to follow increasing percentages until higher concentrations are reached. At higher concentrations the system becomes lipophilic in nature and the titration reflects the solubilization of water rather than salicylic acid. This explanation is borne out by the pronounced decrease in apparent solubilizing power shown with the less polar additives (*n*-pentanol curve, Fig. 2). These phenomena correlate well with Winsor's theory of intermicellular equilibria (9) which explains phase changes observed in proceeding from a lipophilic to a hydrophilic dispersion medium.

As the ratio of salicylic acid to polysorbate 80 is reduced, greater amounts of water can be tolerated by the system until a ratio is obtained which is completely water miscible. The highest ratio at which this occurs is termed the critical miscibility ratio (*CMR*). This ratio is not meaningful at very low salicylic acid concentrations approaching the limit of water solubility of salicylic acid; however it gives a good indication of the minimum amount of solubilizer needed for water-miscible combinations. Table I which records the *CMR* values for a series of added alcohols reveals that, although alcohols such as ethanol may assist in solubilization at the lower concentrations of water (Fig. 1), the effect disappears at the *CMR*. In the series of monohydric alcohols, the decrease in *CMR* follows their polarity, the decrease paralleling the decrease in dielectric constant. The constancy of the *CMR* for the polyhydric alcohols is remarkable in the light of the report by Coles (6) that glycerin markedly increases the solubilizing power of polysorbate 80 for vitamin A palmitate in the formulation of water-miscible vitamin A preparations. Possibly the different results observed in this study for salicylic acid could be due to its semipolar nature while vitamin A palmitate is of much lower polarity. For mixtures of polysorbate 80 and Tween 20 (Table II) the change in *CMR* parallels the relative concentration of the two micelle-forming agents.

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Complex Formation Between Hydrocolloids and Tranquilizers and Hypotensive Agents

By HORACE D. GRAHAM, YEDE MARIE BAKER, and A. N. NJOKU-OBI†

Highly insoluble complexes are formed between hydrocolloids such as carrageenan and sodium carboxymethylcellulose and sodium alginate, and tranquilizers and hypotensive agents such as promazine hydrochloride, chlorpromazine hydrochloride, and reserpine. Meprobamate and hexamethonium chloride form much weaker complexes as evidenced by their failure to produce turbidity with several hydrocolloids tested. Using the promazine hydrochloride–carrageenan (Seakem type 5) interaction as the experimental system, it was shown that maximum interaction occurred at 45° and at a pH of 4.95 in a buffered medium. After intramuscular injection of the carrageenan–promazine hydrochloride complex into rabbits, free promazine was detected in the blood up to six days after administration as contrasted to the disappearance of the free form, similarly administered, after only 24 hours.

RECENTLY, chlorpromazine has gained much attention, not only because of its tranquilizing effect but also because of its interaction with sulfated mucopolysaccharides such as chondroitin sulfate and other biochemical compounds (1–4). Since chondroitin sulfate occurs in dentin and enamel and is produced in excessive quantities in abnormal calcification of the arteries and bone (5, 6), such interactions are important pathologically. In one specific study, Sobel and Burger (6) exploited it to study the mechanism of dentin formation. DeLuca and Kostenbauder (7) mentioned that drugs such as chlorpromazine, promethazine, and tetracaine hydrochlorides are bound by nonionic surfactants but, as far as can be ascertained, a comprehensive study of the binding of tranquilizers and hypotensive agents by hydrocolloids has apparently not been made.

Many tranquilizers, when given in large doses, may cause several undesirable side effects. On the other hand, if small compatible doses are given, the treatment must be repeated at frequent intervals. For these reasons, sustained release dosage forms of many medicinals have become of primary importance.

Chemically, sulfated hydrocolloids like carrageenan, furcellaran, fucoidan, etc., are similar to chondroitin sulfate, are readily available, and are very reactive. Therefore, it was decided to study the interactions of these natural compounds with tranquilizers and hypotensive agents. It was considered that if highly insoluble complexes could be obtained, these studies would provide some basis for the use of some of these hydrocolloids in the development of sustained release forms of the drugs. Since the hydrocolloids are natural, readily available, and highly innocuous products, their introduction into biological systems would present no medico-legal problems.

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EXPERIMENTAL

Reagents

Hydrocolloids.—These are listed in Table I. Unless otherwise stated, all dispersions were prepared as described by Graham and Thomas (8). All calculations were made on a dry weight basis.

Tranquilizers and Hypotensive Agents.—Promazine hydrochloride, 0.5% in distilled water; chlorpromazine hydrochloride, 0.5% in distilled water; Serpasil phosphate (reserpine), 0.1% in distilled water; veratrine sulfate, 0.5% in distilled water; Veriloid, 0.1% in dilute acetic acid; and veratrine alkaloid, 0.1% in dilute acetic acid.

Equipment

The following were used: a Coleman Universal spectrophotometer, model 14; a Beckman pH meter, model G; Pyrex glass-stoppered test tubes; and dialysis membrane, 27/32 dialysis tubing, seamless cellulose.[1]

Procedure

General Survey of the Interaction of Hydrocolloids with Tranquilizers and Hypotensive Agents.—In order to obtain some idea of the reaction of a broad spectrum of hydrocolloids with several tranquilizers and hypotensive agents, a screening program was undertaken. For this, 1–5 ml. of each hydrocolloid suspension was mixed with 2 ml. of the stock solution of each tranquilizer or hypotensive agent and the development or nondevelopment of turbidity noted. The results of this study are summarized in Table I.

Relative Reactivity of Hydrocolloids with Tranquilizers and Hypotensive Agents.—Since in some

[1] From Fisher Scientific Co

cases only slight precipitation was observed in the screening program, more information was sought on the relative reactivity of the hydrocolloids with tranquilizers and hypotensive agents. The sulfated hydrocolloids were previously observed to be precipitated most readily by the drugs in question. Moreover, the turbidity developed was shown to be proportional to the amount of hydrocolloid present. Based on this premise, a scheme was developed for comparing the reactivities of the hydrocolloids.

One to five ml. containing 1–10 mg. of the particular hydrocolloid was mixed with 2 ml. of the stock solution of the particular tranquilizer or hypotensive agent at 45°. The total volume in all cases was 10 ml., attained by adding distilled water where necessary. The reaction was allowed to proceed for 15 minutes, the tubes were cooled to room temperature and the turbidity developed measured at 400 mμ, with a Coleman Universal spectrophotometer, model 14. Since some solutions were appreciably viscous, accompanying control tubes were included by substituting 2 ml. of distilled water for the solution of tranquilizer or hypotensive agent. The actual turbidity (optical density) developed, therefore, was calculated as "the turbidity measured minus the turbidity (if any) of the accompanying control."

In order to compare the reactivities of the hydrocolloids, the turbidity produced by 1 ml. of carrageenan (Seakem type 5) with a particular tranquilizer or hypotensive agent was assigned an arbitrary value of 10. The turbidity produced by 1 mg. of any other hydrocolloid through interaction with the same tranquilizer or hypotensive agent was then compared with the value obtained for carrageenan (Seakem type 5) to give values recorded in Table II.

Influence of Variables on the Interaction.—Several variables such as temperature, pH, buffer normality, interaction time, and the presence of inorganic salts could possibly influence the binding of the drugs by the hydrocolloids. Therefore, these variables were systematically investigated. Unless otherwise stated, the carrageenan (Seakem type 5)-promazine hydrochloride system was used throughout the investigations on variables.

The influence of temperature was assessed by reacting 10 mg. of the hydrocolloid with 2 ml. of the drug solution at 30, 45, and 65° in a total volume of 10 ml. (Where reactions were done at 45 and 65°, the tubes were immediately cooled in an ice water bath after the desired reaction time.) After adjusting to room temperature, the turbidity (optical density) developed was measured at 400 mμ with the Coleman Universal spectrophotometer, model 14. For the establishment of the optimum

TABLE I.—SURVEY OF THE INTERACTION OF HYDROCOLLOIDS WITH TRANQUILIZERS AND HYPOTENSIVE AGENTS[a]

Hydrocolloids	Tranquilizers		Hypotensive Agents			
	Promazine Hydrochloride	Chlorpromazine Hydrochloride	Reserpine	Veriloid	Veratrine Alkaloid	Veratrine Sulfate
Group I.—Sulfated polysaccharides						
Carrageenans (Seakem types 4, 5, 7, 402)	+	+	+	+	+	+
Furcellaran	+	+	+	+	+	+
Sodium kappa carrageenan	+	+	+	+	+	+
Sodium lambda carrageenan	+	+	+	+	+	+
Gigartina acicularis	+	+	+	+	+	+
Gigartina pistillata	+	+	+	+	+	+
Eucheuma cottonii	+	+	+	+	+	+
Eucheuma spinosum	+	+	+	+	+	+
Fucoidan	+	+	+	+	+	+
Agar	+	±	−	−	±	+
Hypnean	+	+	+	+	+	+
Iridophycan	+	+	+	+	±	+
Gelcarin	+	+	+	+	+	+
Group II.—Carboxylic acid type polysaccharides						
Gum karaya	+	+	−	−	±	−
Gum arabic	−	−	−	−	±	±
Gum tragacanth	−	−	−	−	±	+
Gum ghatti	−	−	−	−	+	±
Pectin	+	+	−	−	±	+
Sodium carboxymethylcellulose	+	+	+	+	±	+
Sodium alginate	+	+	+	+	±	+
Group III.—Neutral polysaccharides						
Locust bean gum	−	−	−	−	−	−
Gum guar	−	−	−	−	±	−
Potato starch	−	−	−	−	−	−
Group IV.—Proteins						
Casein	+	+	−	−	+	+
Gelatin	−	−	−	−	±	±
Group V.—Other compounds						
Heparin	+	+	+	+	+	+
Chondroitin sulfate	+	+	+	+	+	+
Polyglucose sulfate	+	+	+	+	+	+

[a] + = precipitation; − = no precipitation; and ± = gelation.

TABLE II.—RELATIVE REACTIVITY OF THE HYDROCOLLOIDS WITH TRANQUILIZERS AND HYPOTENSIVE AGENTS

Hydrocolloids	Tranquilizers: Promazine Hydrochloride	Tranquilizers: Chlorpromazine Hydrochloride	Hypotensive Agents: Reserpine	Hypotensive Agents: Veriloid	Hypotensive Agents: Veratrine Sulfate
Group I.—Sulfated polysaccharides					
Carrageenan Seakem type 5	10.00	10.00	10.00	10.00	10.00
Carrageenan Seakem type 402	10.62	11.20	9.85	14.00	11.40
Furcellaran	5.39	5.80	6.70	5.4	6.2
Sodium kappa carrageenan	10.55	10.20	9.98	10.6	10.8
Sodium lambda carrageenan	16.00	15.80	14.79	13.2	14.6
Viscarin	9.74	10.4	11.94	10.8	11.97
Agar	0.08	0.11	0.46	0.08	0.40
Gelcarin	12.95	11.20	9.98	11.2	9.92
Group II.—Carboxylic acid type polysaccharides					
Gum karaya	1.03	0.98	0.06	0.04	0.26
Gum arabic	0.03	0.01	0.09	0.01	0.04
Gum tragacanth	0.00	0.09	0.00	0.00	0.00
Gum ghatti	0.09	0.08	0.16	0.01	0.01
Pectin	0.04	0.04	0.15	0.04	0.04
Sodium carboxymethylcellulose	0.84	0.82	6.18	2.76	0.06
Sodium alginate	0.48	0.47	0.70	0.82	0.36
Group III—Neutral polysaccharides					
Locust bean gum	0.01	0.04	0.71	...	0.03
Gum guar	0.34	0.05	0.33	...	0.60

interaction time at 45°, mixtures of the hydrocolloid and the drug in a total volume of 10 ml. were allowed to interact for the periods shown in Table III and the optical density measured as described above. Studies on the effects of pH were done by adding 2 ml. of 0.1 *M* phosphate buffers of varying pH levels to test tubes containing a suspension of 10 ml. of the hydrocolloid and 2 ml. of the drug solution. The total volume was always 10 ml. The mixtures were incubated at 45° for 15 minutes and, after cooling to room temperature, the turbidity that developed was measured at 400 mμ. The final pH of each system was checked with a Beckman model G pH meter. Since the drugs gave precipitates with buffer salts in the alkaline range, all experimentation was restricted to the acid range. Any influence of buffer molarity was ascertained by adding varying amounts of 1.0 *M* phosphate buffer, pH 4.95, to test tubes containing 1 ml. of the hydrocolloid suspension and 2 ml. of the drug solution. After making the volume 10 ml., the turbidity developed at 45° for 15 minutes was measured as described above.

Since pharmaceutical formulations may contain certain salts, the influence of some of the more common salts on the intensity of the interaction was

TABLE III.—INFLUENCE OF VARIABLES ON THE INTERACTION OF CARRAGEENAN[a] WITH PROMAZINE HYDROCHLORIDE

Variable	Absorbance at 400 mμ: Immediately Measured	Absorbance at 400 mμ: After Cooling and Brought to Room Temperature
Temp., (° C.)		
30	0.153	0.155
45	0.220	0.324
65	0.142	0.318
Reaction Time, Min. at 45° C.		
0	0.180	0.300
5	0.200	0.320
10	0.205	0.320
15	0.215	0.320
30	0.215	0.325
36	0.210	0.370
45	0.195	0.370
90	0.240	0.330
Buffer Molarity		
0.01	...	0.198
0.05	...	0.198
0.10	...	0.200
0.30	...	0.185
0.50	...	0.181
0.70	...	0.145
pH	Promazine Hydrochloride Bound μM/mg. of Carrageenan (Seakem type 5)	
5.50 (unbuffered)	...	1.40
2.55	...	1.15
2.85	...	1.18
4.45	...	1.20
4.95	...	1.55

[a] Seakem type 5.

TABLE IV.—INFLUENCE OF ADDED SALTS ON THE INTERACTION OF CARRAGEENAN[a] WITH PROMAZINE HYDROCHLORIDE AT 45° C.

Salt Added as Chloride	Tolerance Level, *M*
Monovalent	
Li^+	4.25×10^{-4}
K^+	3.0×10^{-4}
NH_4^+	6.4×10^{-4}
Na^+	5.0×10^{-4}
Divalent	
Mg^{++}	2.5×10^{-4}
Ba^{++}	5.0×10^{-4}
Cd^{++}	1.1×10^{-4}
Zn^{++}	1.4×10^{-4}
Sr^{++}	1.4×10^{-4}
Mn^{++}	2.5×10^{-4}
Ca^{++}	1.9×10^{-4}
Trivalent	
Al^{+++}	1.2×10^{-8}
Fe^{+++}	1.0×10^{-4}

[a] Seakem type 5.

assessed. This was done by adding varying amounts of each salt solution to the reaction tubes and measuring the optical density as described above. Duplicate control tubes containing no added salt were included in each experiment. The tolerance level of each salt was established in a manner similar to that employed by Graham and Thomas (8). It was taken as "That maximum final concentration (the concentration of salt in the 10 ml. of reaction medium) of salt which did not cause a significant difference (in this case ±1%) in the absorbance as compared to a control tube to which no salt was added." The results are summarized in Table IV.

Maximum Binding Capacity of Hydrocolloids for the Tranquilizers and Hypotensive Agents.—More detailed quantitative data on the binding process were obtained by determining the maximum binding capacity of representative hydrocolloids for promazine hydrochloride and reserpine. For this, 5 ml. of the suspension containing 50 mg. of the particular hydrocolloid was allowed to react at 45° for 15 minutes with increasing quantities of the tranquilizer or hypotensive agent, in a centrifuge tube, and with a total volume of 10 ml. After cooling to room temperature, the reaction mixture was centrifuged at 1500 r.p.m. for 10 minutes. One milliliter of the supernatant was placed in 9 ml. of methyl alcohol and this alcohol mixture again centrifuged as above. One milliliter of this supernatant was then used for the colorimetric determination of the particular tranquilizer or hypotensive agent. The amount of the drug bound was calculated as "the amount added minus the amount found free in the supernatant."

Colorimetric Methods for Determination of Tranquilizers and Hypotensive Agents. Promazine was determined according to the method of Leach and Crimmin (9). Reserpine was determined by the method of Indemans, *et al.* (10).

Calculation of the Maximum Binding Capacity.—The maximum amount of promazine hydrochloride and reserpine bound per gram of three representative hydrocolloids was established from a Langmuir type plot as employed by Kennon and Higuchi (11) and Patel and Kostenbauder (12). By plotting the reciprocal of the amount of drug bound per unit of hydrocolloid on the Y axis *vs.* the reciprocal of the amount of free drug in the system, and extrapolating to zero concentration of the drug, a line which cuts the Y axis was obtained. The Y intercept gives a measure of the maximum binding capacity (13). The results are summarized in Table V and representative graphs are shown in Figure 1.

Solubility of the Promazine Hydrochloride–Hydrocolloid Complexes.—Since the promazine hydrochloride–carrageenan complex was so readily formed and seemed highly insoluble, it was deemed useful to study its solubility in solutions of sodium chloride and of hydrochloric acid. The method used was similar to that employed by Antonopoulous, *et al.* (14), in studying detergent complexes and Bridger, *et al.* (15), in studying metal ammonium phosphate complexes. For this, 5 ml. of a dispersion containing 50 mg. of carrageenan (Seakem type 5) and 5 ml. of a 0.5% solution of promazine hydrochloride were allowed to react at 45° for 30 minutes in 15-ml. graduated centrifuge tubes. After cooling, the mixture was centrifuged at 2500 r.p.m. for 10 minutes and the supernatant carefully poured off. The precipitate was washed five times with distilled water and centrifuged after each washing. The precipitate from the centrifuge tubes was added to consecutive 200 × 15 Kimax test tubes and quantities of 4 M sodium chloride or 2 N hydrogen chloride added to give the final normalities shown in Table VI. The final volume was 20 ml. in all cases attained by adding distilled water where necessary. The tubes were shaken on a Burrell wrist action shaker at 27° and, at the intervals shown, 3 ml. of the

Table V.—Maximum Binding Capacity of Hydrocolloids for Promazine Hydrochloride and Reserpine at 45° C.

Hydrocolloids	μM of Drug Bound/Gm. of Hydrocolloid: Promazine Hydrochloride	Reserpine
Carrageenan (Seakem type 5)	1500	666.0
Na carboxymethylcellulose	244	540.5
Pectin	14.3	238.0

Table VI.—Solubility of the Promazine Hydrochloride-Carrageenan[a] Complex in Sodium Chloride and Hydrochloric Acid at 27° C.

	% of Bound Promazine Released After 1 Hr.	6 Hr.	12 Hr.	24 Hr.
	Sodium Chloride (Final Molarity)			
0.0 (H_2O)	4.000	4.01	4.17	4.87
0.2	26.52	29.46	30.55	29.17
0.4	29.65	31.63	34.72	38.88
1.6	38.74	40.42	48.61	43.75
	Hydrochloric Acid (Final Normality)			
0.0 (H_2O)	4.00	4.01	4.17	4.87
0.01	7.00	7.82	8.33	13.18
0.1	18.46	20.01	23.61	30.00
0.2	25.20	26.52	30.55	36.11
0.5	24.62	29.80	38.19	58.33
1.0	40.80	48.60	57.08	65.29
2.0	56.40	60.00	79.17	83.33

[a] Seakem type 5.

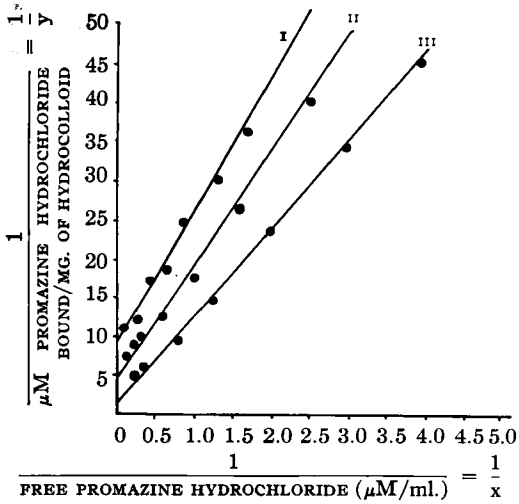

Fig. 1.—Langmuir type plot of data to determine maximum binding capacity of hydrocolloids for promazine hydrochloride. I = pectin; II = sodium carboxymethylcellulose; and III = carrageenan (Seakem type 5).

mixture centrifuged and one ml. of the supernatant assayed for promazine released from the complex. The results are shown in Tables VI and VII. For comparison, the promazine hydrochloride–sodium carboxymethylcellulose complex was also studied.

Experiments on the Promazine Hydrochloride–Carrageenan Complex.—Batch quantities were prepared by using a 200-ml. suspension containing 200 mg. of carrageenan (Seakem type 5), preincubated at 45° for 30 minutes. One hundred milliliters of a solution containing 2 Gm. of promazine hydrochloride was similarly preincubated. At the end of the preincubation period, both the hydrocolloid suspension and the promazine hydrochloride solution were mixed in a 1000-ml. Erlenmeyer flask and allowed to interact for 30 minutes at 45°. The mixture was placed in a cold room at 4° overnight and filtered. The filtrate was collected and carrageenan added to precipitate any promazine in it. If precipitation occurred, the cooling and filtration processes were repeated. The precipitates were pooled and washed with distilled water to remove any unreacted hydrocolloid. This was then followed by washing 5 times with absolute methyl alcohol. The precipitate was then collected, placed in a Petri dish and dried overnight in a vacuum oven at 75°. After this, the mass was pulverized in a mortar and stored in a vial.

Titration of the Complex.—In order to determine the promazine content of the complex 10 mg. was placed in a 25-ml. volumetric flask and enough concentrated sulfuric acid added to dissolve it. Water was added to make it up to volume. From this, 1 ml. was taken, further appropriately diluted with distilled water and the promazine content determined colorimetrically by the method of Leach and Crimmin (9).

Sustained Release of the Promazine from the Complex *in vivo*.—In order to ascertain if sustained release of the promazine from the carrageenan (Seakem type 5)–promazine complex could be achieved, free promazine, and the suspending medium, respectively, was injected intramuscularly into individual rabbits. Sterile physiological saline and a 1:5:4 mixture of ethyl alcohol, glycerin, and water were used as suspending media in the separate experiments. Samples of blood were drawn at intervals from 4 hours to 6 days and analyzed for promazine by the method of Leach and Crimmin (9). Simultaneously, changes in the picture of the white blood cells were followed. The results are summarized in Table VIII.

TABLE VII.—SOLUBILITY OF PROMAZINE HYDROCHLORIDE-SODIUM CARBOXYMETHYLCELLULOSE COMPLEX IN SODIUM CHLORIDE AND HYDROCHLORIC ACID AT 27° C.

	% of Bound Promazine Released After— 1 Hr.	6 Hr.	12 Hr.	24 Hr.
Sodium Chloride (Final Molarity)				
0.0 (H_2O)	10.6	11.4	12.6	14.3
0.2	68.0	71.4	78.0	88.4
0.4	72.0	78.0	87.0	94.3
0.8	75.2	81.6	90.0	98.0
1.6	80.2	87.6	91.2	98.4
2.0	81.6	90.2	94.3	98.7
Hydrochloric Acid (Final Normality)				
0.0 (H_2O)	10.6	11.4	12.6	14.3
0.01	20.6	25.0	28.6	30.2
0.10	28.0	38.0	40.8	53.0
0.20	40.8	46.0	65.8	76.5
0.50	68.7	85.6	93.0	94.6
1.00	72.4	86.2	94.5	96.2
2.00	76.0	87.0	94.3	96.4

RESULTS AND DISCUSSION

From Tables I and II it is clear that the sulfated polysaccharides are the most reactive hydrocolloids. The low reactivity of agar may be partly explained by the fact that its sulfation is still not well established (16, 17). The carboxylic acid type polysaccharides are next in order of reactivity. Of these, sodium carboxymethylcellulose is one of the more reactive. In the experiments with sodium carboxymethylcellulose, all reagents were dissolved in distilled water to avoid precipitation of the hydrocolloid itself at low pH levels of the medium (17). The neutral polysaccharides were the least reactive.

Of the several variables which affect the interaction of promazine hydrochloride with carrageenan (Seakem type 5), the pH of the interaction medium is the most important. In the alkaline range, the tranquilizer will precipitate in the absence of the hydrocolloid, hence only the acid range was investigated. Maximum interaction occurs at pH 4.95, and as the pH decreases the amount of promazine bound per mg. of the hydrocolloid decreases. At a final pH of 2.25, binding of the drug by the hydrocolloid was still appreciable since ionization of the sulfate group is not drastically impeded even at this low final pH. A similar pattern would be expected for the other sulfated hydrocolloids. However, the carboxylic acid type hydrocolloids would be more severely influenced since at pH levels of 2.5–3.0, sodium carboxymethylcellulose, in particular, would precipitate in the absence of the tranquilizer.

The temperature has a great influence on the interaction as exemplified by the promazine hydrochloride–carrageenan (Seakem type 5) interaction. In the acid range (pH 4.95) turbidity is a good measure of the intensity of the interaction and it was used to assess this influence. At 30°, the turbidity developed was lower than at 45° or at 65°. Apparently, at this temperature (30°) ionization of both interactants may not be complete. The great sensitivity of the system to temperature as indicated

TABLE VIII.—DETECTION OF PROMAZINE IN BLOOD OF RABBIT AFTER INTRAMUSCULAR INJECTION OF PROMAZINE HYDROCHLORIDE AND THE CARRAGEENAN-PROMAZINE COMPLEX

Time Lapse After Injection, Hr.	Promazine Detected in Blood After Injection of Carrageenan[a] Promazine Complex μg./ml. of Blood	Promazine Hydrochloride μg/ml. of Blood
0.0	0.0	0.0
4.0	2.0	2.64
12.0	3.07	4.5
18.0	...	1.9
24.0	3.55	0.0
48.0	3.47	...
72.0	1.29	...
96.0	0.95	...
144.0	0.47	...

[a] Seakem type 5.

TABLE IX.—EFFECT OF THE INTRAMUSCULAR INJECTION OF THE CARRAGEENAN[a]-PROMAZINE COMPLEX ON THE PICTURE OF THE WHITE BLOOD CELLS OF THE RABBIT

Time After Injection, Hr.	Variations in Count of White Blood Cells				
	Neutrophils, %	Monocytes, %	Basophils, %	Eosinophils, %	Lymphocytes, %
0.0	26.8	12.0	0	5.0	54.0
4.0	38.0	12.5	0	3	46.0
12.0	19.0	34	0	0	46.0
18.0	...	...	...	...	...
24.0	45.0	29.5	1.0	2	22.5
48.0	29.0	30.5	0	0	20.5
72.0	30.0	32.0	2	0	36.0
96.0	41.0	19.0	2	3	36
144.0	...	...	...	...	...

[a] Seakem type 5.

by the measurements made immediately (before cooling) and those made after cooling to room temperature indicate that, apparently, forces other than the ionic type can participate in the binding process. Since the turbidity developed at 45° was essentially the same as that at 65° (after cooling), 45° was chosen for all later experiments.

Under the experimental conditions employed, at 45° the maximum interaction occurred after 30 minutes. Despite this, a heating time of 15 minutes was selected to avoid any possibility of hydrolysis of the hydrocolloid due to prolonged heating, and to facilitate rapidity of experimentation when many samples were involved. The degree of interaction at 45° for 15 minutes remained constant for as long as 4 hours, hence comparisons based on the time–temperature selection are not invalid.

Final buffer molarities of 0.01–0.10 resulted in no drastic change in the interaction as evidenced by the turbidity measurements. Therefore, where buffers were used, a final molarity of 0.01 was selected.

Table IV summarizes the influence of salts on the promazine hydrochloride–carrageenan (Seakem type 5) interaction. On a molar basis, the order of tolerance of the added salts was found to be monovalent > divalent > trivalent. Severe interference of the salts is due to competition for the binding sites of the hydrocolloids. The trivalent cations will actually precipitate carrageenan and other hydrocolloids, even at relatively low concentrations.

Although only the promazine hydrochloride–carrageenan (Seakem type 5) system was investigated in detail, it is to be expected that the other systems would be similarly affected by the variables listed. As noted with the precipitation with alkaloids (8), lambda carrageenan was always more reactive than kappa carrageenan. This is in line with chemical data which show that the lambda fraction is much richer in ester sulfate content than the kappa fraction (17).

The solubility data recorded in Tables VI and VII indicate that the promazine hydrochloride complexes of carrageenan (Seakem type 5) and sodium carboxymethylcellulose are highly insoluble in sodium chloride and hydrochloric acid. Relatively, the sodium carboxymethylcellulose complex is more soluble than the carrageenan complex in both sodium chloride and hydrochloric acid. This insolubility renders them potentially suitable as sustained release forms of this and possibly other drugs. The need for complexing agents for the development of such depot forms of drugs has recently been clearly emphasized by Hirscher and Miller (18).

Analysis of the data recorded in Table VIII indicate that the promazine hydrochloride–carrageenan (Seakem type 5) complex can serve as a depot form of the drug. The prolonged presence of the drug in the blood of the rabbit leads to the conclusion that the drug was released from the complex. Contrastingly, the disappearance of drug administered as the "free form," after 24 hours, indicates the further superiority of the complex where sustained release is desired. Le Blanc (19) reported that a single injection of chlorpromazine caused a drop in the eosinophils, leucocytes, and platelets in the blood of male albino rats. This effect, in their opinion, was not a direct result of hypothermia. Korst (20) has reviewed several cases of agranulocytosis caused by phenothiazine derivatives including chlorpromazine and promazine. It was noted that in some cases the neutrophils decreased drastically or even vanished and that the total leucocyte count severely decreased. Since promazine hydrochloride is very closely related to chlorpromazine hydrochloride, it was decided to investigate the blood picture changes, if any. The variations in the blood cell picture shown in Table IX, may be considered as further evidence that the promazine was being released from its hydrocolloid complex. Besides the possibility of their use for development of sustained release forms of drugs, the effect of these hydrocolloids on the availability of drugs and other chemicals in pharmaceutical preparations must also be emphasized. It has been shown that gums (hydrocolloids) will lower the availability of tetracycline antibiotics.

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Technical Articles

Technique of Implanting Permanent Electrodes in Cats for Chronic Stimulation and Observation of EEG and Behavioral Effects

By ZOLA P. HOROVITZ and MAY-I CHOW

A procedure for preparing cats with permanently implanted electrodes is described. This technique, which has been used to successfully prepare over 50 of these animals, employs fixation with dental cement and the use of a direct contact connection on the top of the head. The procedure is quick, inexpensive, and very productive. This makes it quite useful in both industrial and academic pharmacological, physiological, and psychological research.

SINCE Hess' pioneer work on stimulation of the unrestrained, unanesthetized cat (1), techniques for stimulating and lesioning or for recording of electrical and behavioral phenomena in the active animal have become useful to the pharmacologist, physiologist, and psychologist. Bradley and Elkes (2), Horovitz and Chow (3), and many others, have used cats with permanently implanted electrodes (P.I.E. cat) for correlating the effects of drugs upon electrical and behavioral responses. This type of preparation has also been used for various types of chronic stimulation by Killam and Killam (4), Doty (5), and Horovitz, *et al.* (6).

The literature, unfortunately, reveals only a few complete descriptions of the techniques involved in preparing these P.I.E. animals. The techniques used by Hess, ingenious for his time, have been outdated by technical advancements. Knowles (7) in 1951 described a method of implantation that employed a base-plate assembly. This technique requires a large craniotomy and the building of a complicated base-plate head assembly for each cat. Bradley and Elkes (8) in 1953 described an excellent procedure for implanting deep and cortical electrodes but the leads were threaded under the skin of the neck and out the animal's back. This required the animal to carry a bulky harness on its back. Delgado (9) has described a method of implanting multipolar needle electrodes; unfortunately his technique also requires a bulky harness. Delgado (10) has expertly reviewed various implantation procedures and the effects of long term stimulation and recording in animal brains.

Our laboratory has explored various aspects of many techniques for preparing the P.I.E. cat. We feel that the procedure described below is the quickest, easiest, and most productive we have tested. It requires very little laboratory preparation; every item is commercially available, and the animal requires very little maintenance care. We have successfully used this procedure to prepare over 50 P.I.E. cats within the past two years.

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The authors wish to thank Dr B. N. Craver for his advice and encouragement and Dr. L. J. Brannick and Mr. T. Waldron for their invaluable technical assistance.

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Technical Articles

Technique of Implanting Permanent Electrodes in Cats for Chronic Stimulation and Observation of EEG and Behavioral Effects

By ZOLA P. HOROVITZ and MAY-I CHOW

A procedure for preparing cats with permanently implanted electrodes is described. This technique, which has been used to successfully prepare over 50 of these animals, employs fixation with dental cement and the use of a direct contact connection on the top of the head. The procedure is quick, inexpensive, and very productive. This makes it quite useful in both industrial and academic pharmacological, physiological, and psychological research.

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Our laboratory has explored various aspects of many techniques for preparing the P.I.E. cat. We feel that the procedure described below is the quickest, easiest, and most productive we have tested. It requires very little laboratory preparation; every item is commercially available, and the animal requires very little maintenance care. We have successfully used this procedure to prepare over 50 P.I.E. cats within the past two years.

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MATERIALS

Deep Electrodes.—The deep electrodes (Fig. 1-B) are of the bipolar concentric type and consist of insulated 0.0117 in. platinum-tungsten wire inserted into 23-gauge stainless steel hypodermic tubing. The inter-polar distance varies between 0.6–0.8 mm. Each tip exposure is approximately 0.3 mm. A patch wire is soldered onto the tubing about 5 mm. from the top. The length from the tip to the patch solder joint varies, depending upon the electrode placement. Generally, the length is equal to 5 mm. plus the distance between electrode tip and surface of cortex, as determined from the cat brain atlas (11). This allows enough extra length on the electrode for cementing to the skull surface. The tubing is insulated with three or four coats of BC-341 Dolphon epoxy resin varnish and the wire with two coats of Formvar insulation.[1]

Surface Corticals.—The surface cortical electrodes are 5-mm. long stainless steel rivets with the heads removed. Flexible lead wires are soldered onto the top of the rivets. The rivets are 1.5 mm. thick and insulated with Insl-X varnish,[2] except at the tip (Fig. 1-C).

Ground and Support Screws.—The ground wire is usually three or four entwined strands of platinum wire forced into one of the temporal muscles or wound around one of the support screws. These screws are No. 4-36 × 1/4 in. machine screws.

Electrode Connectors.—The electrode connector is an Amphenol Blue Ribbon 14 pin connector #57-40140 (Fig. 1-A). This connector was found to be the easiest to handle and solder of all the connectors tested.

Cement.—All electrodes and the connector are fixed to the skull with Caulk Grip dental cement. This cement is a quick drying powder-liquid resin that has proved to be very easy to handle. To increase the gripping effect, Caulk dental primer is painted on the skull surface prior to cementing.

METHOD

Most of our cats have been adult female cats of more than 2.0 Kg., deprived of their ovaries and uteri. We have successfully used six male cats, but in general find the spayed female a much more stable and tractable animal. At least two weeks prior to implantation, the cats were immunized against feline pneumonitis and feline infectious enteritis (distemper).

The cats are anesthetized intraperitoneally with 35 mg./Kg. of pentobarbital sodium. Sterile operating procedures have been found to be unnecessary but the electrodes are sterilized overnight under ultraviolet light and all the instruments are soaked in iodine solution[3] prior to use. The head and neck are shaved and painted with antiseptic solution. The animal is placed into the stereotaxic instrument and a midline incision is made in the scalp. The incision is approximately 5 cm. in length and ends just behind the occipital protuberance. The temporal muscles are reflected to give the desired skull exposure. Small pock marks

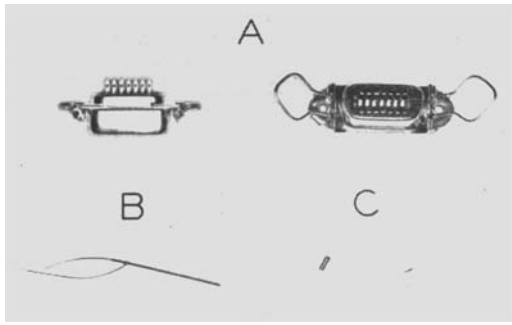

Fig. 1.—*A* = electrode connector; *B* = deep bipolar electrode; *C* = cortical electrode.

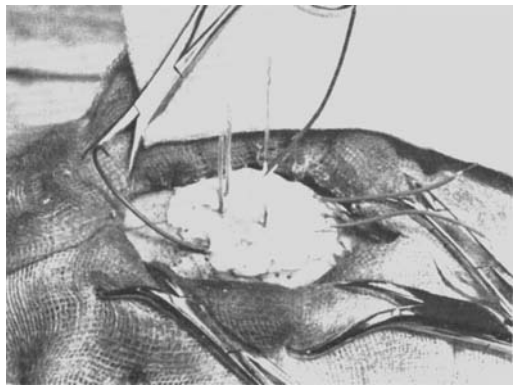

Fig. 2.—Deep and cortical electrodes cemented onto top of skull.

are made on the skull with a No. 1/2 burr on a dental drill. These marks serve as additional gripping points for dental cement and help to hold the electrodes in place. The previously zeroed electrodes[4] are then placed into the holders and moved to the appropriate lateral and anterior-posterior positions prior to insertion. The electrodes are then lowered to a point just above the surface of the skull and the skull is marked with pencil just below the electrodes. After all the electrode skull-entry points have been marked, they are drilled with a No. 4 burr. Care is taken not to damage the dura. After drilling, any bleeding between the bone plates is stopped by plugging the hole for a few minutes with Gelfoam. After each deep electrode has been inserted into the brain, the skull surface around it is carefully dried with cotton swabs and primer and a small amount of cement applied. About 5 minutes is allowed for the cement to dry before the electrode holder is removed (Fig. 2).

The holes for the corticals are drilled with a No. 5 burr and those for the supporting screws with a No. 8 burr. After these are in place they are also cemented to the skull (Fig. 2). Using an Oryx miniature soldering iron, the previously tinned ends of the electrodes are then soldered onto the appropriate connector pins (Fig. 3). All soldered joints are tested for mechanical strength and elec-

[1] Finished electrodes available from Lehigh Valley Electronics, Fogelsville, Pa.
[2] Insl-X Corporation, Ossining, N. Y.
[3] Weladol disinfectant, Allied Laboratories, Indianapolis, Ind.
[4] Most of our experiments employed the Baltimore Instrument Company stereotaxic apparatus, model L, Baltimore, Md. The brochure with the instrument provides full and adequate instructions for zeroing electrodes.

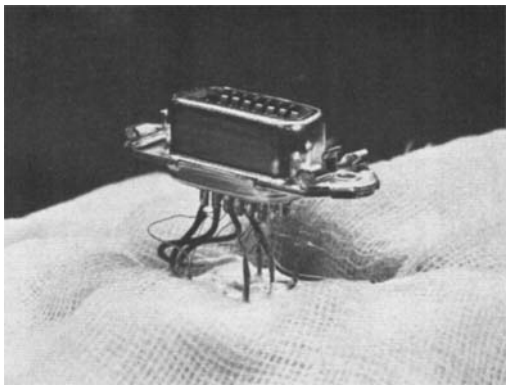

Fig. 3.—Electrode connector showing solder connections with electrodes from brain.

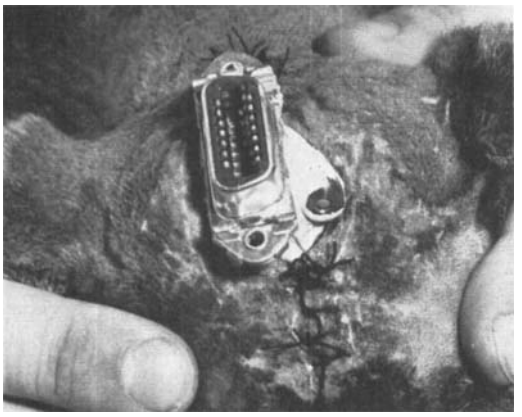

Fig. 4.—Finished preparation with venous cannula.

trical conductivity. The wires are then carefully coiled to avoid shorting and the connector is placed as close to the skull surface as possible. The connector and wires are then cemented to the skull. Care must be taken at all times not to abrade the insulation on the lead wires. The incision is then closed with No. 2-0 surgical silk thread and the animal removed from the stereotaxic instrument. The cat is usually placed in a warm cage overnight and is, in most cases, alert and hungry the following morning. Figure 4 illustrates a completed preparation. The rubber diaphragm on the right is cemented to the skull and attached on the inside to polyethylene tubing which is brought down under the skin and inserted into the jugular vein for subsequent intravenous injections. Figure 5 is an X-ray of another P.I.E. cat.

After a few preliminary scratchings, the cats do not disturb the plug. Any infection that develops under the skin surrounding the connector can be kept under control by cleaning and washing with iodine solution. More severe infections have been successfully treated with a triamcinolone-antibiotic combination ointment.[5]

To record or stimulate, an amphenol male connector No. 57-30140, attached by cable to the stimulator and electroencephalograph apparatus, is connected to the female connector on the animal's head. The connectors are durable, and since the contact is a wiping one there is always a clean contact surface.

It has been our experience that this technique of fixation with dental cement and the use of a direct contact connection on the top of the head is more practical and less bulky, has a longer duration and is better tolerated than any other procedure presently described in the literature.

[5] Mycolog ointment, E. R. Squibb and Sons, Div. of Olin Mathieson Chemical Corp., New York, N. Y.

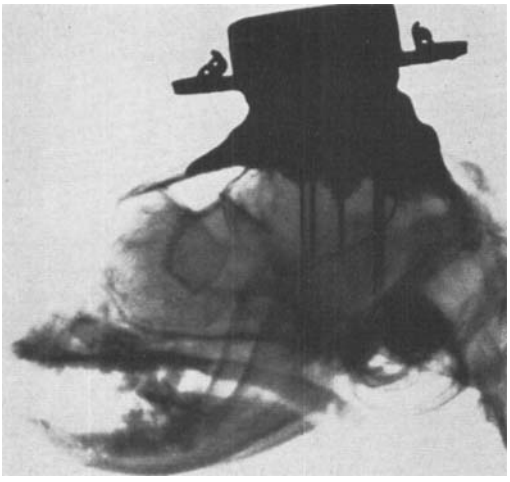

Fig. 5.—Sagittal X-ray of finished preparation.

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Spectrophotometric Determination of Three Sulfa Drugs in Combination

By S. M. MODY and R. N. NAIK

The principle of simultaneous equations has been applied to a tertiary mixture containing three sulfa drugs. A rapid procedure for the spectrophotometric determination, without prior separation, of sulfacetamide, N^1-benzoylsulfanilamide, and sulfathiazole in combination has been developed.

WHERE the individual amounts of three sulfa drugs appearing in combination with each other in a product are to be estimated, the official procedures (1, 2) utilize paper strip chromatography to first separate each drug and then colorimetric procedures to estimate the individual amounts of the components. The official procedures are quite time-consuming and not so accurate. There is a need, therefore, for a method which will permit the quick and accurate estimation of sulfacetamide, N^1-benzoylsulfanilamide, and sulfathiazole in combination with each other in a water-dispersible cream base.

Marzys (3) has developed a procedure for the individual estimation of the components of sulfathiazole, sulfamerazine, and sulfadiazine mixtures utilizing a combination of colorimetric and spectrophotometric procedures. Since no specific color reaction was known for any of the three sulfas studied, their ultraviolet absorption spectra in 0.1 *N* hydrochloric acid were used to determine whether or not the amounts of the individual sulfas could be estimated from the absorption at three wavelengths and solving simultaneous equations with three unknowns (4).

EXPERIMENTAL

Apparatus: Beckman spectrophotometer model DU 4700 with 1-cm. matched quartz cells was used. Cell correction was applied where necessary. Unless otherwise mentioned the measurements were done against 0.1 *N* hydrochloric acid as blank.

Reagents: Hydrochloric acid, A.R. grade, solvent ether, reagent grade, sulfacetamide U.S.P. XV (5), sulfathiozole N.F. X (6), N^1-benzoyl sulfanilamide, supplied by Bengal Immunity Co. Ltd., conforming to the specifications of Basu & Sikdar (7).

The absorption spectra for the three sulfas were determined at 220, 235, and 280 mμ (see Fig. 1). They conformed to Beer's law between 5 and 25 mcg./ml.; the absorption being additive. The absorptivities of the three compounds, determined using 1-cm. quartz cells and 0.2-mm. slit width, are given in Table I.

By solving the following three simultaneous equations from the absorbances at the indicated wavelengths, one obtains the concentrations of the three ingredients:

$$460\ a + 465\ b + 345\ c = 1000 \times A_{220}$$

$$56.5\ a + 584\ b + 106.5\ c = 1000 \times A_{235}$$

$$167.5\ a + 189\ b + 489\ c = 1000 \times A_{280}$$

where a = concentration of sulfacetamide, b = N^1-benzoylsulfanilamide, and c = sulfathiazole, expressed as mg. per 100 ml. of solution.

Analysis of Known Synthetic Mixtures.—Known mixtures were prepared by taking suitable aliquots of solutions of individual components. The acidity in the final solution was maintained at 0.1*N*. The absorbances were measured at 220, 235, and 280 mμ and the content of each component calculated using simultaneous equations. The results are given in Table II.

Analysis of the Cream Containing Sulfacetamide, N^1-Benzoylsulfanilamide, and Sulfathiazole.—About 1 Gm. of the cream (containing about 100 mg. of total sulfonamides) was mixed with a little solvent ether to get a good dispersion and transferred to a 250-ml. separator with the aid of about 50 ml. of

TABLE I.—ABSORPTIVITY VALUES (E 1%, 1 CM.)

Wavelength, mμ	220	235	280
Sulfacetamide	460	56.5	167.5
N^1Benzoylsulfanilamide	465	584	189
Sulfathiazole	345	106.5	489

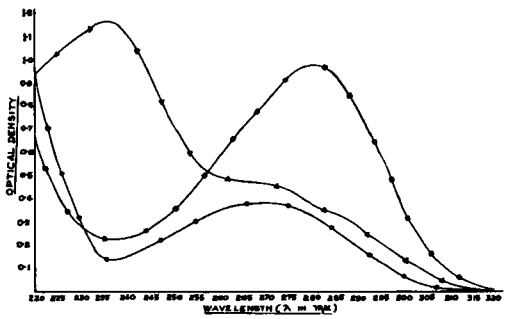

Fig. 1.—Absorption spectra determined for the three sulfa drugs studied. Sulfacetamide —●—●—, N'—benzoylsulfanilamide —▲—▲—, sulfathiazole —■—■—, (concn., 20 mcg./ml.)

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TABLE II.—RESULTS WITH KNOWN MIXTURES, MG./100 ML.

No.	Sulfacetamide		N^1-Benzoyl sulfanilamide		Sulfathiazole		Total	
	Found	Added	Found	Added	Found	Added	Found	Added
1	0.810	0.800	0.589	0.600	0.637	0.600	2.036	2.000
2	0.404	0.400	1.005	1.000	0.630	0.600	2.039	2.000
3	0.366	0.400	0.812	0.800	0.803	0.800	1.981	2.000
4	1.227	1.200	0.409	0.400	0.368	0.400	2.004	2.000

TABLE III.—ASSAY RESULTS OF OINTMENT

No.	Sulfacetamide, %	N^1-Benzoylsulfanilamide, %	Sulfathiazole, %	Total, %
1	2.765	3.749	3.363	9.877
2	2.947	3.744	3.343	10.034
3	2.799	3.840	3.406	10.045
Average	2.837	3.777	3.371	9.985
Claim	2.860	3.700	3.420	9.980

solvent ether. The suspension was extracted with four 50-ml. portions of 1 *N* hydrochloric acid. The combined acid extracts were washed in a 500-ml. separator with 25 ml. of solvent ether and filtered through a Whatman No. 41 filter paper into a 500-ml. volumetric flask. The filter was washed and the volume made up with 1 *N* hydrochloric acid. A 10-ml. aliquot was pipetted into a 100-ml. volumetric flask and the volume made up with distilled water. A 200-ml. portion of 1 *N* acid was similarly treated to prepare the blank.

The absorbances of the sample solution at 220, 235, and 280 mμ, measured against the blank, were used in the calculations as above to obtain the results in Table III.

CONCLUSION

A spectrophotometric procedure has been developed for rapid and fairly accurate determination of three individual sulfa drugs in combination with each other without prior separation. Though the combination is not the one very frequently used, the procedure could, perhaps, be extended to other combinations as well.

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Synthesis of Methylglyoxal-bis-guanylhydrazone-C^{14}

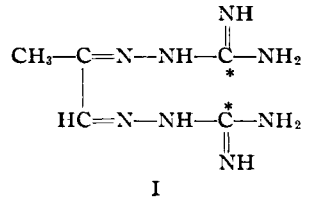
By VINCENT T. OLIVERIO and CHARLENE DENHAM

Methylglyoxal-bis-guanylhydrazone (methyl GAG), an antitumor agent in current animal and clinical trials, has been synthesized with isotopic carbon (C^{14}) in excellent yield and radiochemical purity for pharmacological studies.

NUMEROUS reports have recently appeared relating the inhibitory effects of guanylhydrazones, particularly methylglyoxal - bis - guanylhydrazone (methyl GAG), on various animal and human neoplasms (1–7). To facilitate studies on the metabolic fate and mechanism of action of these agents in man and animals, we have synthesized radioactive methyl GAG-C^{14} (I) in excellent yield and radiochemical purity.

Methyl GAG may be prepared by the reduction of nitroguanidine with zinc dust in acetic acid to aminoguanidine (8), followed by condensation with pyruvaldehyde (9). The aminoguanidine may also be prepared, alternatively, as originally described for pilot plant production (8), by methylation of thiourea with dimethyl sulfate followed by hydrazinolysis of the S-methylisothiuronium sulfate.

For the preparation of methyl GAG-C^{14} on the milligram scale, the latter procedure was chosen because: (*a*) appreciable quantitites of zinc salts were coprecipitated with addition of sodium bicarbonate after reduction of nitroguanidine, even in the presence of ammonium chloride, resulting in an impure product; (*b*) the specific activity (mc./mmole) of the commercially available C^{14}-guanidine nitrate was only one-fourth as high as the less expensive C^{14}-thiourea; and finally; (*c*) the overall reaction yield (30%) of the first procedure was appreciably lower than the yield (70%) of the alternate



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Received April 27, 1962, from the Clinical Pharmacology and Experimental Therapeutics Section, Medicine Branch, National Cancer Institute, Bethesda 14, Md.
Accepted for publication May 25, 1962.
Guanidine-C^{14}, 1,1′-(methylethanedilidenedinitrilo)dihydrochloride monohydrate, is the full chemical name. The abbreviated name, methyl GAG-C^{14}, is employed throughout the text.
The thiourea-C^{14} was obtained from New England Nuclear Corp., Boston, Mass.
Pyruvaldehyde (43%) was obtained through the courtesy of Union Carbide Chemicals Co., New York, N. Y.

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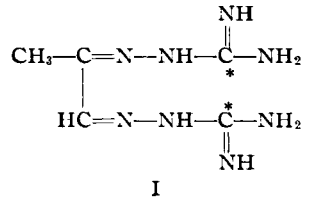
NUMEROUS reports have recently appeared relating the inhibitory effects of guanylhydrazones, particularly methylglyoxal - bis - guanylhydrazone (methyl GAG), on various animal and human neoplasms (1–7). To facilitate studies on the metabolic fate and mechanism of action of these agents in man and animals, we have synthesized radioactive methyl GAG-C^{14} (I) in excellent yield and radiochemical purity.

Methyl GAG may be prepared by the reduction of nitroguanidine with zinc dust in acetic acid to aminoguanidine (8), followed by condensation with pyruvaldehyde (9). The aminoguanidine may also be prepared, alternatively, as originally described for pilot plant production (8), by methylation of thiourea with dimethyl sulfate followed by hydrazinolysis of the S-methylisothiuronium sulfate.

For the preparation of methyl GAG-C^{14} on the milligram scale, the latter procedure was chosen because: (*a*) appreciable quantitites of zinc salts were coprecipitated with addition of sodium bicarbonate after reduction of nitroguanidine, even in the presence of ammonium chloride, resulting in an impure product; (*b*) the specific activity (mc./mmole) of the commercially available C^{14}-guanidine nitrate was only one-fourth as high as the less expensive C^{14}-thiourea; and finally; (*c*) the overall reaction yield (30%) of the first procedure was appreciably lower than the yield (70%) of the alternate



I

Received April 27, 1962, from the Clinical Pharmacology and Experimental Therapeutics Section, Medicine Branch, National Cancer Institute, Bethesda 14, Md.
Accepted for publication May 25, 1962.
Guanidine-C^{14}, 1,1′-(methylethanedilidenedinitrilo)dihydrochloride monohydrate, is the full chemical name. The abbreviated name, methyl GAG-C^{14}, is employed throughout the text.
The thiourea-C^{14} was obtained from New England Nuclear Corp., Boston, Mass.
Pyruvaldehyde (43%) was obtained through the courtesy of Union Carbide Chemicals Co., New York, N. Y.

method. Thus, in combination with several minor modifications in manipulation and apparatus for adaptation to the semimicro scale, the dihydrochloride salt of methyl GAG-C^{14} was prepared in 99% radiochemical purity with a specific activity of 1.95 mc./mmole.

PROCEDURE

S-Methylisothiuronium-C^{14} Sulfate.—The method was adapted from "Organic Syntheses" (10). In a 20-ml. round bottom flask fitted with a condenser and a soda-lime trap were mixed 10 mmoles of thiourea-C^{14} [50.6 mg. of thiourea-C^{14} (15 mc./mmoles) and 710 mg. of unlabeled thiourea] and 1 ml. of water. To this was added, dropwise, 1 ml. (10 mmoles) of freshly distilled dimethyl sulfate and the mixture was gently refluxed for 1 hour. The reaction mixture was concentrated on a steam bath in a stream of nitrogen to approximately one-half the original volume. Upon cooling, the solidified contents were triturated with several milliliters of ethanol, filtered, and washed with ether. The white solid material was suspended in 10 ml. of a warm mixture of 95% ethanol and ether (1:1), filtered, and washed with ether. The first crop of crystals weighed 406 mg. and melted, with decomposition, at 242°. Concentration of subsequent filtrates to an oil, precipitation with ether, and recrystallization from a mixture of 95% ethanol and either (1:1) yielded an additional 783 mg. of product melting at 242–244°, with decomposition. The total yield was 1.188 Gm. (85%).

Aminoguanidine-C^{14} Bicarbonate.—The procedure was an adaptation of a pilot plant scale reaction in "Organic Syntheses" (8). Hydrazine sulfate (1.095 Gm., 8.4 mmoles) suspended in 3 ml. of water was solubilized by the measured, dropwise, addition of 12 *N* sodium hydroxide solution until neutral to congo red. A second equal volume of sodium hydroxide solution was then added. This hydrazine solution was rapidly added through a reflex condenser to a solution of 1.188 Gm. (4.2 mmoles) of S-methylisothiouronium-C^{14} sulfate and 1 ml. of water in a 20-ml. round-bottom flask. The reaction mixture was stirred magnetically for 1 hour at room temperature. Evaporation of the solution *in vacuo* on a steam bath to one-half the original volume yielded a negligible amount of hydrated sodium sulfate crystals which were removed by filtration. The filtrate was warmed to 30° and glacial acetic acid was added to pH 5. Approximately 1 Gm. of sodium bicarbonate was added to the solution which was stirred at room temperature for 1 hour or until precipitation was complete. The white aminoguanidine bicarbonate salt was filtered, washed with cold water, and dried *in vacuo* at room temperature. The product weighed 1.043 Gm. (90.5%) and melted, with decomposition, at 175°.

Methylglyoxal-bis-guanylhydrazone-C^{14}.—The procedure of Thiele (9) was adapted. A mixture of 1.043 Gm. (7.6 mmoles) of aminoguanidine-C^{14} bicarbonate, 1 ml. of 12 *N* hydrochloric acid, and 0.5 ml. of 95% ethanol was refluxed 30 minutes in a 20-ml. round-bottom flask to give white needles of the hydrochloride salt. To the cooled reaction mixture were added, dropwise, 0.85 ml. (6 mmoles) of 43.2% pyruvaldehyde (partially decolorized by treatment with Darco-G60) and 2 ml. of 95% ethanol. The mixture was stirred 45 minutes at ice-bath temperature. The reaction mixture was poured into 50 ml. of acetone to give a white flocculent precipitate of the bis-guanyldhydrazone dihydrochloride monohydrate. The product was filtered and washed with cold acetone and ether. After drying *in vacuo* at room temperature, the pure compound weighed 982 mg. (3.57 mmoles, 93%) and melted at 256–257°, with decomposition.

The specific activity of the pure radioactive product (1.95 mc./mmoles) was determined by liquid scintillation counting of 0.2-ml. aliquots of a standard aqueous solution (100 mcg./ml.) dissolved in 18 ml. of 30% methanol in toluene containing 0.3% of 2,5-diphenyloxazole and 0.01% of 1,4-bis[2-(5-phenyloxazolyl)]benzene. An absolute counting efficiency of 63% was obtained when samples were counted in plastic bottles. When glass counting bottles were used there was a rapid loss of counts in the samples due to the absorption of the methyl GAG on the surface of the glass container (11).

The radiopurity of the product was assayed by high voltage paper electrophoresis and by paper chromatography in two solvent systems, followed by autoradiography of papers and counting of radioactivity in all spots (12). High voltage electrophoresis (40 v./cm.) of the drug on Whatman No. 3MM paper at pH 3.5 in 0.05 *M* ammonium formate buffer, resulted in a single spot migrating toward the cathode and containing more than 99% of the radioactivity of the load. No radioactive contaminant was detected. In a descending system of *n*-propanol, 1 *N* hydrochloric acid, and water (3:1:1) methyl GAG-C^{14} had an R_f of 0.36 and contained more than 99% of the total load radioactivity. In butanol, 95% ethanol, and water (4:1:1), the compound had an R_f of 0.15 and no radioactive contaminent. In both systems the R_f values for the radioactive compound were identical with those of authentic methyl GAG as located by ferricyanide nitroprusside reagent (13). Lastly, the ultraviolet absorption spectrum at pH 6 of the radioactive methyl GAG was in agreement with authentic methyl GAG.

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Occurrence of a Weak Anti-inflammatory Substance in *Simaba cedron* Seed

By E. ROY HAMMARLUND

Ethanol and aqueous extracts of *Simaba cedron* seeds contain a weak, nonspecific, and relatively toxic anti-inflammatory substance.

It was suggested that *Simaba cedron* seed, *Simaroubaceae*, possibly contained an anti-inflammatory or antiarthritic agent because a 5% extract of the powdered seeds in 70% ethanol had been used orally in Denmark for rheumatoid arthritis with some apparent success (1).

Previous investigations on the plant have been few and the results have been contradictory. Nauck and Picado (2) reported limited success with injections of an alcoholic extract of cedron seed as a treatment for malaria, whereas Spencer, *et al.* (3), found no antimalarial activity present. Gauckler (4) described the use of the cotyledons as a remedy for snakebite and fever, whereas Bonsmann (5) reported that an extract of cedron had no antisnakebite activity. Krebs and Rüber (6) isolated cedrin, a nonalkaloid or glycosidal bitter principle, and reported that it is probably a partially hydrated naphthalene derivative similar to santonin. However, no tests were made for any biological activity. Polonsky (7) isolated two nonalkaloidal cedron fractions, cedronine and cedronyline, but likewise did not test for biological activity. Moreover, Wall, *et al.* (8), in their numerous surveys of plants for the presence of steroidal sapogenins did not investigate *Simaba cedron*.

The object of this initial investigation was to make several crude extracts of the powdered dried cedron seeds by employing various solvents and to biologically screen these extracts on rats for the possible presence of any anti-inflammatory principle. The finding of any appreciable amount of activity would then have been followed by an intensive search for, and the isolation of, the particular biologically active ingredient. However, none of the extracts yielded a substance sufficiently active to warrant further and more complete investigation. Nevertheless, because there may be some value in an essentially negative report, the experimental procedures and findings of this study are here reported very briefly.

EXPERIMENTAL

Extraction Procedure.—Dried seeds which had been collected in South America were verified by botanists of S. B. Penick and Co. and E. R. Squibb and Co. The seeds were finely powdered in a grinder and this powder was used for all extractions. Approximately 500 Gm. of the powdered seeds were used for each of the individual extractions by the following solvents: petroleum ether, anhydrous ethyl ether, ethanol, and water. Three or four repeated extractions were made with each solvent on a separate batch of seeds using a high-speed blender at room temperature. No attempt was made to obtain a complete extraction with any solvent. The solvents were evaporated in a rotary film evaporator without heat and the following amounts of extractives were obtained: with petroleum ether, 500 Gm. of powdered seeds yielded 36.6 Gm. of a yellow oil; with anhydrous ethyl ether, 500 Gm. of powdered seeds yielded 48.8 Gm. of a viscous yellow oil which contained a small amount of white solids; with ethanol, 500 Gm. of powdered seeds yielded 27.1 Gm. of a sticky dark yellow solid; with distilled water, 400 Gm. of powdered seeds yielded 37.8 Gm. of a dried yellow solid which could be powdered.

These extracts were dried in a vacuum desiccator and the vials were tightly stoppered under nitrogen. The samples were sent to the Squibb Institute for Medical Research for routine screening for the presence of anti-inflammatory activity.

Biological Screening Tests and Results.—The four extracts were assayed in both intact and adrenalectomized male rats implanted with cotton pellets. Results of these studies indicate that the petroleum ether soluble and anhydrous ethyl ether soluble extracts were inactive even at daily subcutaneous doses of 10 mg. per animal. The ethanol soluble extract was lethal at 10 mg. in the adrenalectomized animal. This ethanol extract induced a slight anti-inflammatory effect in the intact rat at daily doses of 0.4 mg. and 2 mg. The water soluble extract was lethal in the intact rat at a 10-mg. dose and had a weak anti-inflammatory effect at a 4-mg. daily dose, but was inactive at lower doses. In additional studies in intact animals the water soluble extract partially prevented (28%) the hind limb edema induced by the parenteral administration of dextran.

DISCUSSION

The results indicate that the extracts of the seeds are highly toxic and have only a weak, nonspecific anti-inflammatory activity. A large number of toxic or irritating substances such as turpentine could produce similar effects in these same tests. Therefore, there is little justification for continuing the search for an anti-inflammatory agent in this plant. No attempt was made to identify the slightly active substance.

Since the aqueous solutions of the powdered seeds foamed so copiously during the extraction and evaporation, it was suspected that there were probably saponins, sapogenins, or protein material present. Using a DuNouy tensiometer, a 1% aqueous solution of the aqueous extract was found to lower the surface tension of distilled water a total of 28.1 dynes/cm. at room temperature. Furthermore, in order to find out if this aqueous solution was strongly hemolytic to red blood cells, two drops of fresh human capillary blood were added to flasks containing 25 ml. of 0.1%, 1.0%, and 5.0% aqueous solutions of the extract each in 0.6%, 0.75%, and 0.9% sodium chloride solutions. The solutions were stoppered,

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The author is indebted to the Squibb Institute for Medical Research for carrying out the biological screening tests.

mixed by inversion, and allowed to stand one hour at room temperature and for an additional 24 hours at approximately 5°. None of the solutions caused any hemolysis of the erythrocytes by the end of the 25 hours. This indicates that the saponin content is not great and the extract is not strongly hemolytic.

The powdered seeds were further investigated for the presence of alkaloids by employing the routine acidic-aqueous and alkaline-chloroform partition extraction procedure twice and then testing the aqueous solution with Mayer's reagent and, likewise, the chloroform solution on a spot plate with Erdmann's, Froehde's, and Mandelin's reagents. All tests were negative, which indicates the absence of alkaloids in cedron seeds.

SUMMARY

1. *Simaba cedron* seeds contain a weak, nonspecific, and relatively toxic anti-inflammatory substance.

2. An aqueous extract of the seeds is nonhemolytic to erythrocytes and does not contain alkaloids.

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Application of Solvent Extraction and Anion-Exchange Chromatography to the Determination of Sodium Pantothenate in Pharmaceutical Products

By T. PANALAKS

A spectrophotometric method, developed for calcium *d*-pantothenate, was demonstrated to be equally applicable to the sodium salt.

A CHEMICAL method for the determination of calcium *d*-pantothenate in pharmaceutical products was recently developed in this laboratory (1). The method was based in part on an extraction with benzyl alcohol in the presence of a salting-out agent and on an anion exchange chromatography. Since different salts were shown to affect the partition coefficient of the pantothenate in the solvent extraction, it was necessary to investigate the applicability of the method to sodium *d*-pantothenate which is used in some pharmaceutical products. The possibility of using the calcium salt as a reference standard in the determination of the sodium form was also studied by comparing the relative absorbance produced by the reaction product of the two salts.

EXPERIMENTAL

Preparation of Sodium Pantothenate.—A 5.0-Gm. quantity of calcium *d*-pantothenate was dissolved in a minimum volume of distilled water and 21.5 ml. of a 5% solution of sodium carbonate was added with stirring. The solution was let stand overnight in a refrigerator, then filtered on a No. 1 Whatman. The precipitate was washed with about 350 ml. distilled water. The combined filtrate and washings was adjusted to pH 6.0, and made up to 500 ml. with distilled water.

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Helpful discussions concerning this paper by Dr. J. A. Campbell and statistical assistance of Mr. J. Malcolm Airth are gratefully acknowledged.

Recovery Test.—Amounts of 1.235, 2.470, and 4.940 ml. of the prepared solution were each added to 25 ml. of a liquid multivitamin preparation. These amounts were equivalent to 0.5, 1.0, and 2.0 mg. of sodium *d*-pantothenate per ml. of the liquid sample. The determinations were made in duplicate on aliquots of 10 ml. of the solutions after being diluted to a 100 ml. with distilled water. Calcium *d*-pantothenate was the reference standard and a factor of 1.0124 was used for the conversion of calcium *d*-pantothenate to its equivalent of the sodium salt.

RESULTS AND DISCUSSION

To compare the behavior of the two salts, 5 mg. of the calcium salt and an equivalent weight of the prepared sodium salt were subjected to the procedure (1). The assays were conducted in three replicates on different days. The results, presented in Table I, indicated that although there was significant variation between assays for the sodium salt there was no significant difference on the average between the absorbance of the reaction products of the two salts. No apparent destruction of the pantothenate radical due to hydrolysis was observed. Since it was shown previously that the presence of amino acids did not significantly affect the final absorbance readings (1), and that an equal response of both forms of the pantothenate was obtained in this experiment, it was concluded that β-alanine was not formed before the pantothenate was subjected to the complete analytical procedure.

The results of the recovery test of sodium *d*-

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The results of the recovery test of sodium *d*-

TABLE I.—RELATIVE ABSORBANCE[a] OF REACTION PRODUCTS OF CALCIUM AND SODIUM PANTOTHENATE

Determination No.	Assay 1 CaPan	Assay 1 NaPan Equivalent	Assay 2 CaPan	Assay 2 NaPan Equivalent	Assay 3 CaPan	Assay 3 NaPan Equivalent
1	145	129	149	141	145	156
2	144	132	147	148	149	152
3	138	131	144	141	143	146
4	133	137	141	153	163	150
5	146	125	154	137	138	160
Mean	141.2	130.8	147.0	144.0	147.6	152.8
Std. Error	2.5	2.0	2.2	2.9	4.2	2.4

[a] Absorbance × 100 per mg.

TABLE II.—RECOVERY OF SODIUM *d*-PANTOTHENATE ADDED TO A COMPLEX VITAMIN PREPARATION

Sodium *d*-Pantothenate, mg./ml. Added	Found	Recovered
0	0.25	...
0.50	0.83	0.58
1.00	1.38	1.13
2.00	2.20	1.95

pantothenate added to a liquid elixir containing a mixture of the B vitamins and yeast extracts, as shown in Table II, indicated a recovery of 97–116%.

Samples of typical multivitamin preparations containing sodium *d*-pantothenate were analyzed, using calcium *d*-pantothenate as a reference standard. Three of these samples were in a lyophilized form. The results, shown in Table III, indicated a reasonable mean overage of 20% of the declared potency.

It may be concluded that the spectrophotometric method developed for calcium *d*-pantothenate is equally applicable to the determination of its sodium salt in pharmaceutical products, and that calcium *d*-pantothenate may be used as a reference standard.

TABLE III.—SODIUM *d*-PANTOTHENATE CONTENT OF MULTIVITAMIN PREPARATIONS

Sample	Mg. per ml. or Vial — Label Claim	Found
1. Injectable, lyophilized, B vitamins,[a] C, gentisic acid ethanolamide	20	22.7
2. Injectable, lyophilized, B vitamins,[a] C	5	7.13
3. Injectable, lyophilized, B vitamins,[a] C	5	5.20
4. Liquid drops, B vitamins,[a] A, C, D	8.33	10.2

[a] Including thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, niacinamide, vitamin B_{12}, and sodium *d*-pantothenate.

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Thin-Layer Chromatographic Identity Test for Steroids in Sesame Oil Preparations

By B. P. KORZUN and S. BRODY

A thin-layer chromatographic method is described as a rapid identity test for methandrostenolone,[1] estradiol dipropionate,[2] testosterone propionate,[3] desoxycorticosterone acetate,[4] and aldosterone acetate in sesame oil preparations. Preliminary extractions are not necessary to obtain desired results.

IT WAS desirable to obtain a rapid method for identifying steroids and decomposition products in sesame oil preparations. Attempts to apply paper chromatography failed because the sesame oil interfered, and the low concentration of steroid present did not give discernible spots with the usual reagents. Thin-layer chromatography, a technique described by Stahl (1, 2), using silica gel G as the adsorbent gave good results. The steroid is well separated from the oil and is easily detected with a modified Le Rosen reagent (3). The time required to complete the test is one to two hours.

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Accepted for publication August 21, 1962.

[1] Marketed as Dianabol by CIBA Pharmaceutical Co.
[2] Marketed as Ovocylin by CIBA Pharmaceutical Co.
[3] Marketed as Perandren by CIBA Pharmaceutical Co.
[4] Marketed as Percorten by CIBA Pharmaceutical Co.

EXPERIMENTAL

Apparatus.—The apparatus for coating the glass plates and other accessories is commercially available from Research Specialties Co., Richmond, Calif.; Arthur H. Thomas Co., Philadelphia, Pa.; and Desaga, Heidelberg, Germany. The adsorbent used is silica gel G.[5]

Preparation of Plates.—A slurry of 30 Gm. of silica gel G and 60 ml. of water is prepared and poured into applicator and spread over the plates.

[5] Manufactured according to specifications of E. Stahl by Merck, A. G., Darmstadt, Germany, and available through Research Specialties Co. and Terra Chemicals, Inc., New York.

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3	138	131	144	141	143	146
4	133	137	141	153	163	150
5	146	125	154	137	138	160
Mean	141.2	130.8	147.0	144.0	147.6	152.8
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Samples of typical multivitamin preparations containing sodium *d*-pantothenate were analyzed, using calcium *d*-pantothenate as a reference standard. Three of these samples were in a lyophilized form. The results, shown in Table III, indicated a reasonable mean overage of 20% of the declared potency.

It may be concluded that the spectrophotometric method developed for calcium *d*-pantothenate is equally applicable to the determination of its sodium salt in pharmaceutical products, and that calcium *d*-pantothenate may be used as a reference standard.

TABLE III.—SODIUM *d*-PANTOTHENATE CONTENT OF MULTIVITAMIN PREPARATIONS

Sample	Mg. per ml. or Vial — Label Claim	Found
1. Injectable, lyophilized, B vitamins,[a] C, gentisic acid ethanolamide	20	22.7
2. Injectable, lyophilized, B vitamins,[a] C	5	7.13
3. Injectable, lyophilized, B vitamins,[a] C	5	5.20
4. Liquid drops, B vitamins,[a] A, C, D	8.33	10.2

[a] Including thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, niacinamide, vitamin B_{12}, and sodium *d*-pantothenate.

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Thin-Layer Chromatographic Identity Test for Steroids in Sesame Oil Preparations

By B. P. KORZUN and S. BRODY

A thin-layer chromatographic method is described as a rapid identity test for methandrostenolone,[1] estradiol dipropionate,[2] testosterone propionate,[3] desoxycorticosterone acetate,[4] and aldosterone acetate in sesame oil preparations. Preliminary extractions are not necessary to obtain desired results.

IT WAS desirable to obtain a rapid method for identifying steroids and decomposition products in sesame oil preparations. Attempts to apply paper chromatography failed because the sesame oil interfered, and the low concentration of steroid present did not give discernible spots with the usual reagents. Thin-layer chromatography, a technique described by Stahl (1, 2), using silica gel G as the adsorbent gave good results. The steroid is well separated from the oil and is easily detected with a modified Le Rosen reagent (3). The time required to complete the test is one to two hours.

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[1] Marketed as Dianabol by CIBA Pharmaceutical Co.
[2] Marketed as Ovocylin by CIBA Pharmaceutical Co.
[3] Marketed as Perandren by CIBA Pharmaceutical Co.
[4] Marketed as Percorten by CIBA Pharmaceutical Co.

EXPERIMENTAL

Apparatus.—The apparatus for coating the glass plates and other accessories is commercially available from Research Specialties Co., Richmond, Calif.; Arthur H. Thomas Co., Philadelphia, Pa.; and Desaga, Heidelberg, Germany. The adsorbent used is silica gel G.[5]

Preparation of Plates.—A slurry of 30 Gm. of silica gel G and 60 ml. of water is prepared and poured into applicator and spread over the plates.

[5] Manufactured according to specifications of E. Stahl by Merck, A. G., Darmstadt, Germany, and available through Research Specialties Co. and Terra Chemicals, Inc., New York.

The plates are then activated in an oven at 80° for 2 hours, and stored over a desiccant in an air-tight container.

Sample Application.—The samples are applied as a streak 2.5 cm. wide, 2.5 cm. apart, and 1.5 cm. from the bottom of the plate. This is accomplished with a micropipet just above the point of application and allowing the solution to be deposited uniformly as the pipet is passed along the 2.5 cm. length. The sample is applied in a volume sufficient to deposit 100 mcg. of the steroid on the plate. An equal volume of sesame oil used to prepare the sample is run as a reference and a 100 mcg. steroid reference sample is also applied. The concentration of steroid in the sesame oil preparation ranges from 0.5 mg./ml. to 50 mg./ml. Therefore, the reference samples of sesame oil must be applied at a volume equal to the volume required to apply 100 mcg. of steroid in the sample preparation.

Solvent Systems.—The developing chambers are lined with filter paper to saturate the chambers. The solvent for testosterone propionate and desoxycorticosterone acetate is chloroform. The solvent for estradiol dipropionate is benzene-methylene chloride (4:6); for aldosterone acetate the solvent is chloroform-ethyl acetate (7:3); and for methandrostenolone the solvent is chloroform-methanol (98:2). The length of development is 15 cm. from the point of application for all solvent systems. After the first development the solvent is dried and developed again.

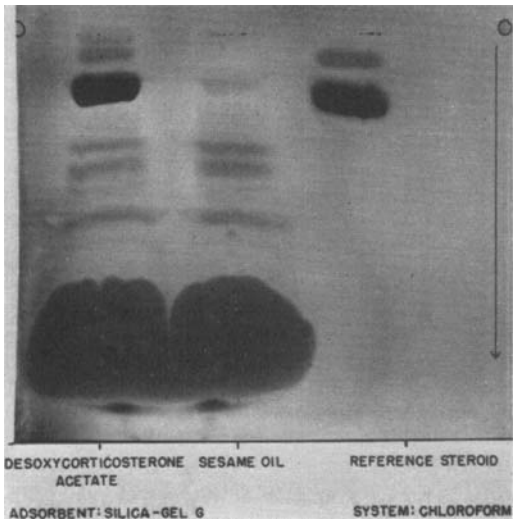

Fig. 1.

Detection.—The steroids are revealed by spraying the plate with a modified Le Rosen reagent (3). The reagent is prepared by dissolving 0.8 ml. formaldehyde (37%) in 10 ml. sulfuric acid and diluted with 3 ml. water. After being sprayed, the plate is heated in an oven at 80° for 5 to 10 minutes. The steroids produce visible colored spots and also produce fluorescent spots under an ultraviolet lamp (3660 Å.).

RESULTS

Figure 1 shows a typical separation of the steroid in sesame oil. The reference steroid shows another slower moving band which can be detected in the oil preparation. Also, another band is detected at the origin in the sample which is not detected in the sesame oil or the reference steroid and which could be a decomposition product of the steroid. Therefore, it is necessary to run a sesame oil reference sample to determine which bands are due to the steroid. Table I lists the R_f values and the colors produced after spraying with formalin reagent. The identity test can be accomplished in one to two hours.

TABLE I.

Steroid	R_f Value[a]	Color	
		Visible	Fluorescent
Methandrostenolone	0.40	Brown	Salmon pink
Estradiol dipropionate	0.45	Red	Orange
Testosterone propionate	0.40	Dark green	Pale pink
Desoxycorticosterone acetate	0.15	Blue	Red
Aldosterone acetate	0.30	Dark yellow	Yellowish green

[a] Value for steroid in solvent described.

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The Editor comments

COMPROMISING PHARMACEUTICAL EDUCATION

Numerous speakers and writers have devoted an almost endless stream of attention in recent years to the changes which have taken place in the field of pharmacy. However, recent developments affecting the orientation of pharmaceutical education make it appear that certain new concepts are now being advanced which seem to call for specific comment.

A few so-called "foreward-looking" pharmacy schools have announced and widely publicized the introduction of revised or new curricula designed to produce pharmacy graduates who will be "better businessmen, better salesmen, and better merchandisers." In some cases abbreviated programs of study are offered for specialized groups, such as prospective medical service representatives. While the specific programs at these schools differ somewhat, they all have one thing in common—commercial courses are being added at the expense of courses in the pharmaceutical sciences.

It remains our firm conviction that it is the primary responsibility of pharmaceutical educators to lead the way in assuring a proper professional-oriented education for tomorrow's pharmacists. These educators must not permit themselves to be led astray into the same pitfalls from which many of today's pharmacists are desperately trying to extricate themselves!

Fortunately, other schools are wisely resisting ill-advised suggestions that they adopt similar programs and philosophies. And although they are implementing well-considered changes in their curricula, which will add to the professional competence and abilities of their graduates, these latter schools are standing by the traditional proposition that it is properly the function of the educational institution to determine what the student should be taught to equip him suitably for his professional pursuits. Consequently, it is particularly offensive that the pressures to reorient educational pursuits toward the commercial aspects are often coming from vocal sources outside the schools of pharmacy, but which nevertheless wish to influence the intended goals of a pharmaceutical education.

Edward G. Feldmann

N. F. XI

FOURTH INTERIM REVISION ANNOUNCEMENT

National Formulary

Eleventh Edition

OFFICIAL FROM MAY 1, 1963

By virtue of the authority conveyed in Chapter III, Article X and Chapter IX, Article V of the By-Laws of the American Pharmaceutical Association, the Council of the Association appointed the Committee on National Formulary, approved the text of this Interim Revision Announcement prepared by the Committee, authorized the printing thereof, and fixed May 1, 1963, as the date upon which it shall become official.

WILLIAM S. APPLE, *Secretary*
Council of the A. Ph. A.

EDWARD G. FELDMANN, *Chairman*
Committee on National Formulary

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AMERICAN PHARMACEUTICAL ASSOCIATION

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JOURNAL OF Pharmaceutical Sciences

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Review Article

X-ray Emission Spectroscopy in Pharmaceutical Analysis

By G. J. PAPARIELLO and W. J. MADER

X-RAY EMISSION spectroscopy (also called X-ray fluorescent spectroscopy) is a rapid, nondestructive, highly sensitive, and precise technique amenable to and applicable to the pharmaceutical control laboratory.

A concise consideration of X-ray fundamentals is presented as an introduction and orientation for those readers with only a superficial knowledge of X-ray methods. The advanced reader is referred to two excellent and very comprehensive books on the subject by Birks (1) and by Liebhafsky and co-workers (2).

FUNDAMENTALS

The Origin of X-rays.—X-rays are produced when fast-moving electrons impinge on matter. The phenomena resulting from the deceleration of such electrons are very complex, and X-rays result from two general types of interaction of the electrons with the atoms of the target material. A high-speed electron may strike and displace a tightly bound electron deep in the atom near the nucleus, thereby ionizing the atom. When a certain inner shell of an atom has been ionized in this manner, an electron from an outer shell may fall into the vacant place, with the resulting emission of an X-ray characteristic of the atom involved. This process produces the characteristic line spectrum of the target element. This production of X-rays is a quantum process similar to the origin of the optical spectra; however, there is a second process in which the high-speed electron is simply slowed down in passing through the strong electric field near the nucleus of an atom. X-radiation produced in this manner is independent of the nature of the atoms being bombarded, and appears as a band of continuously varying wavelength whose lower limit is a function of the maximum energy of the bombarding electrons as shown in Fig. 1.

Continuous Spectrum.—The short-wavelength limit of the continuous spectrum, as seen for tungsten in Fig. 1, is clearly a quantum phenomenon. This short-wavelength limit discovered by Duane and Hunt (3) obeys the relationship

$$\lambda \text{ min.} = \frac{12{,}350}{V} \qquad \text{(Eq. 1)}$$

where λ min. is the minimum wavelength in Ångströms and V is the voltage in volts. This

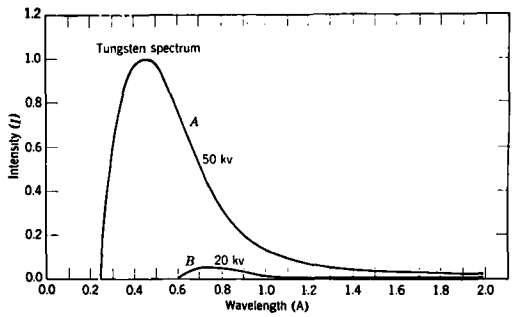

Fig. 1.—The continuous X-ray spectrum. [Reprinted with permission from Liebhafsky, Pfeiffer, Winslow, and Zemany, "X-Ray Absorption and Emission in Analytical Chemistry," Wiley, 1960.]

Received from the Research Department, Ciba Pharmaceutical Co., Summit, N. J.

equation is merely the standard equation relating quantum energy to frequency and is expressed in convenient units. Although the minimum wavelength is independent of the target element, the overall intensity of the spectrum does depend upon the atomic number, Z. This dependency is usually expressed in terms of the frequency, ν, rather than as a function of the wavelength, λ. This relationship is

$$I = AZ(\nu_{max.} - \nu) + BZ^2 \qquad \text{(Eq. 2)}$$

where I is the intensity obtained at a frequency, υ, when $\upsilon_{max.}$ is the maximum frequency (minimum wavelength) obtainable and A and B are constants. One can see from the above expression that the heavier elements will yield more intense primary radiation at all the wavelengths considered.

Characteristic Radiation.—Characteristic radiation from the target element is produced when the high-voltage electrons from a filament have sufficient energy to knock out the inner electrons of the target atoms. Moseley made the first systematic investigation into the production of characteristic radiation (4, 5). He studied the K and L series spectra of 38 different elements by using them successively as the target element and observed that the same type of lines occurred for all of these elements but that they occurred at different wavelengths. From this work Moseley derived the relationship between the atomic number of an element, Z, and the wavelength, λ, at which the characteristic lines of the element occur, or

$$\frac{1}{\lambda} \propto Z^2 \qquad \text{(Eq. 3)}$$

The proportionality constant needed in the above expression is dependent upon which series is being considered. Figure 2 is an illustration of characteristic line dependency on atomic number.

When the applied voltage is sufficient, the filament electrons knock out the inner electrons of the target atoms, and the characteristic lines of the target element will then be superimposed on the continuous radiation curve as in Fig. 3 for molybdenum.

For any element, there is more than one characteristic line because an electron can be ejected from more than one electron shell, and this shell vacancy can be filled by an electron from more than one energy level. Thus the lines of the K series occur if an electron is ejected from a K shell and an electron, in accordance with certain selection rules, from an outer shell fills the vacancy in the K shell. If an L-shell electron fills the K-shell vacancy, the emitted line will be K_α radiation; and if a K-shell vacancy is filled by an M-shell electron, the K_β line is emitted. Similarly, such electronic transitions from outer shells to vacancies in the L shell of atoms lead to the production of the lines of the L series. Figure 4 is a schematic representation of the energy levels for an iron atom showing the transitions that correspond to the four lines of the K spectrum.

BASIC X-RAY EMISSION SPECTROSCOPY SYSTEM

Arrangement and Method of Use.—A brief description of the arrangement of the components of an X-ray emission spectrograph and the manner in which it functions is now in order. It should be noted that although many of the

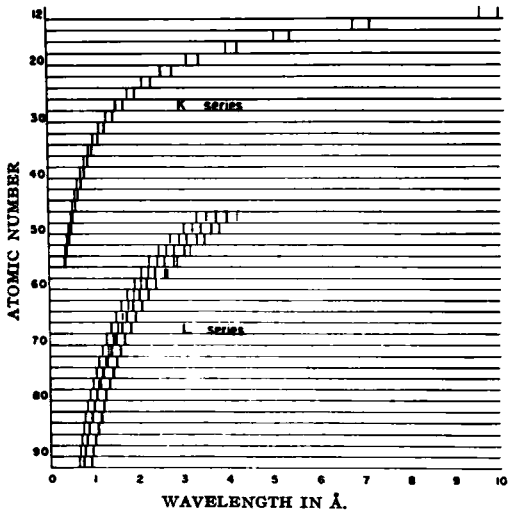

Fig. 2.—The K and L series X-ray lines that are commonly used for X-ray spectrochemical analysis. [Reprinted with permission from Birks, "X-Ray Spectrochemical Analysis," Interscience, 1959.]

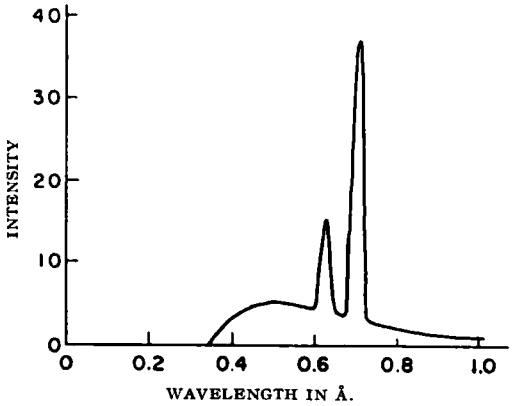

Fig. 3.—The characteristic K series lines of molybdenum superimposed on the continuous spectrum. [Reprinted with permission from Birks, "X-Ray Spectrochemical Analysis," Interscience, 1959.]

components are similar, the arrangement described here for X-ray fluorescent work is different from that used in other X-ray techniques such as diffraction and absorption.

A schematic representation of the arrangement used for fluorescent X-ray spectroscopy is shown in Fig. 5. The main components are *A*, the primary X-ray tube; *B*, the sample; C, the collimator; *D*, the analyzing crystal; and *E*, the detector. The functioning of these components as a unit can best be described by a consideration of the sequence of events which occur when a sample is analyzed. This sequence is as follows:

1. The target material within the X-ray tube is excited, causing the emission of primary radiation. This emitted radiation will consist of characteristic radiation of the target material as well as continuous radiation.

2. This primary radiation strikes the sample with sufficient energy to cause the excitation of the sample. (The foregoing consideration of electron excitation of a primary target and the resulting characteristic lines is applicable to the X-ray photon excitation of the sample as well.) Hence the generation of the characteristic radiation of the elements present in the sample occurs.

3. This characteristic radiation which is emitted in all directions is collected into a parallel bundle by use of the collimator. The collimator is nothing more than a tube filled with parallel metal blades evenly spaced. Thus the radiation emerges from the collimator as a parallel bundle of radiation.

4. This parallel bundle of radiation is polychromatic since each of the elements present in the sample has a different characteristic line. Thus it is necessary to separate this polychromatic radiation into its component parts. This is done by applying the Bragg equation to an analyzing crystal

$$n\lambda = 2d \sin \theta \qquad \text{(Eq. 4)}$$

where n is the order of diffraction, λ is the wavelength of the radiation in Ångströms, d is the interplanar spacing of the analyzing crystal in Ångströms, and θ is the angle formed by the incident radiation and the crystal surface. Rotation of the crystal will change θ, the angle of incidence, and therefore the wavelength of the diffracted radiation will be changed. In this manner the incident polychromatic radiation is separated into its component parts since any one

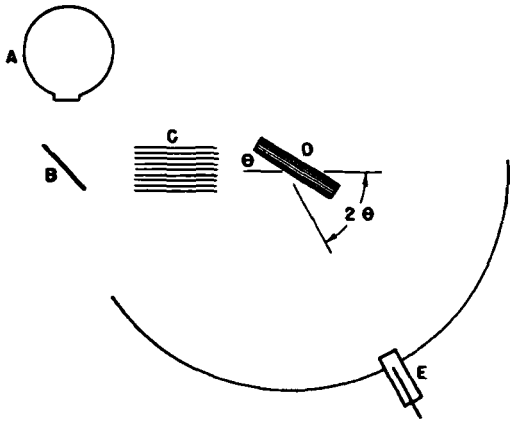

Fig. 5.—Schematic representation of the arrangement used for fluorescent X-ray spectroscopy. [Reprinted with permission from Birks, "X-Ray Spectrochemical Analysis," Interscience, 1959.]

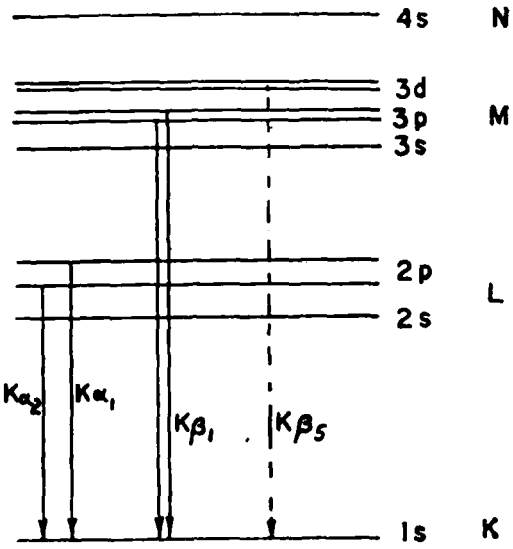

Fig. 4.—Schematic representation of the energy levels for an iron atom showing the transitions that correspond to the four lines of the *K* spectrum. [Reprinted with permission from Birks, "X-Ray Spectrochemical Analysis," Interscience, 1959.]

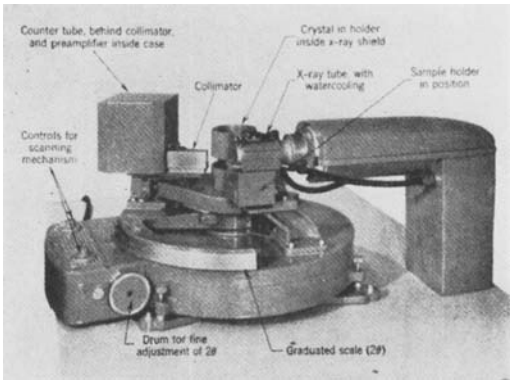

Fig. 6.—XRD-5 D/S spectrogoniometer arranged for X-ray emission work. [Reprinted with permission from Liebhafsky, Pfeiffer, Winslow, and Zemany, "X-Ray Absorption and Emission in Analytical Chemistry," Wiley, 1960.]

crystal rotation position will yield radiation of only one wavelength.

5. The diffracted radiation emerges at an angle of 2 θ with respect to the incident beam and is measured by means of a radiation detector. As stated above, the crystal is rotated, from $\theta = 0°$ to $\theta = 90°$, while the detector is rotated at twice the speed of the crystal so it will always be in position to intercept the diffracted radiation. The goniometer is that part of the equipment which indicates the 2 θ position of the detector. The crystal and detector move as the goniometer moves. The wavelengths of the intercepted X-ray lines indicate the elements that are present in the sample, and the intensities of these lines are an indication of the concentration of each element within the sample.

The actual physical appearance of the instrumentation which performs the above function is depicted in Figs. 6 and 7.

Components.—A more detailed description of several of the components, mentioned just briefly above, is necessary in order for the reader to obtain a more complete understanding of the operation of the X-ray emission spectrograph.

X-ray Tube.—The X-ray tube commonly used in X-ray fluorescent analysis is the hot-cathode or Coolidge tube. These tubes are thoroughly evacuated and sealed. The electrons for excitation are supplied by application of the Edison effect, that is, by emission from a heated filament. A schematic representation of a Coolidge tube is shown in Fig. 8. Two serious drawbacks of this tube are target contamination and the absence of target interchangeability.

Analyzing Crystals.—Crystals which are used as analyzing crystals are not truly perfect crystals but are what are referred to as ideally imperfect crystals. Perfect crystals would give very low diffracted intensities because of primary extinction. Crystals such as lithium fluoride and sodium chloride are ideally imperfect crystals and are commonly used as analyzing crystals.

By inspection of Bragg's equation, Eq. 4, it will be seen that the maximum wavelength that may be diffracted is equal to $2d$, that is, twice the interplanar spacing of the analyzing crystal. Consequently, the wavelengths of the characteristic radiation of the lighter elements become too great for the commonly used crystals. For example, lithium fluoride, $2d = 4.02$ Å, may only be used effectively down to the K lines of potassium, which are at 3.7 Å. Obviously in considering the analysis of lower atomic number elements it is necessary to find suitable crystals with greater d spacings. An example of such a crystal is ethylenediamine ditartrate, $2d = 8.76$ Å, which enables one to work with

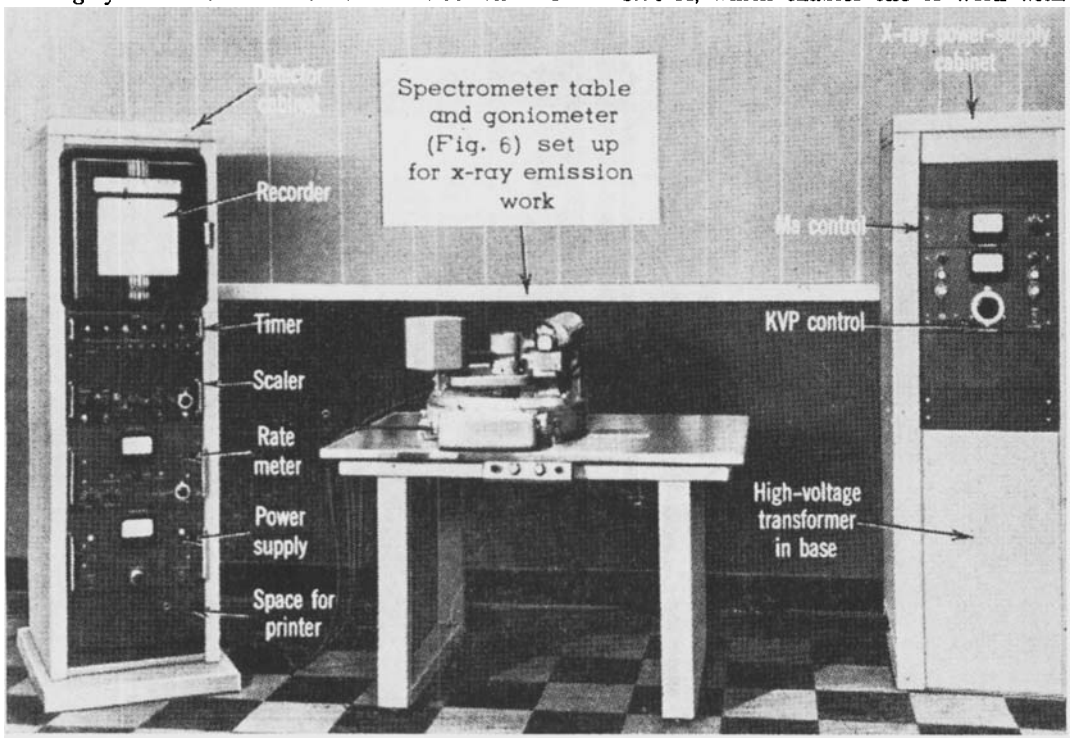

Fig. 7.—XRD-5 D/S spectrometer arranged for X-ray emission work. [Reprinted with permission from Liebhafsky, Pfeiffer, Winslow, and Zemany, "X-Ray Absorption and Emission in Analytical Chemistry," Wiley, 1960.]

aluminum, which has a characteristic line at 8.3 Å.

Detectors.—The detectors in common use for X-ray spectrochemical analysis are the Geiger, proportional, and scintillation counters. These three detectors all operate on the principle of electronic amplification of the energy pulse generated when an X-ray quantum is absorbed. In this way, signals strong enough to operate scaling or integrating circuits are obtained.

It should be noted that photographic film as a means of detection has wide use in X-ray diffraction work but is of little use in emission work.

LIMITING FACTORS OF X-RAY FLUORESCENT ANALYSIS

Fluorescent Yield.—The X-ray fluorescent analysis of all elements is not possible at the present time; however, the analysis of those elements with an atomic number of 23 or above can be performed quite simply and without any special equipment adaptations. On the other hand, the analysis of those elements with an atomic number of 11 or below cannot be assayed by X-ray fluorescent methods. The "light elements," elements with an atomic number of 12 to 22, represent the intermediate range where analysis is possible only after certain difficulties are overcome.

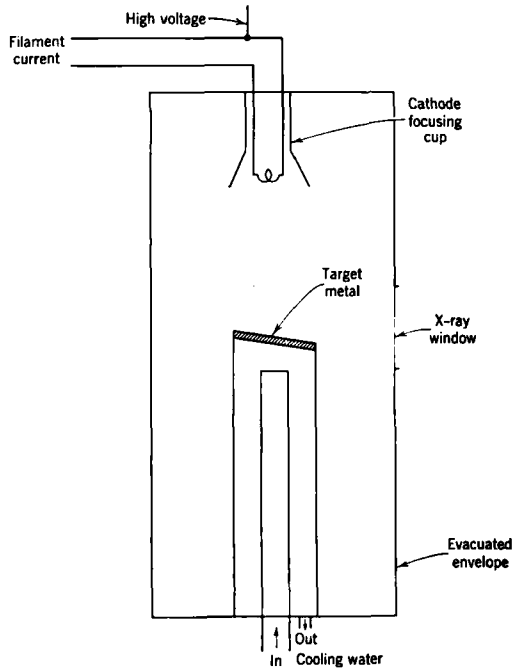

Fig 8.—Schematic diagram of a Coolidge X-ray tube. [Reprinted with permission from Liebhafsky, Pfeiffer, Winslow, and Zemany, "X-Ray Absorption and Emission in Analytical Chemistry," Wiley, 1960.]

The main reason that an elemental distinction must be made, according to atomic number, is that the fluorescent yield of the elements is less than unity, and as the atomic number decreases so does the fluorescent yield as illustrated in Fig. 9. It has been shown that 70% of the K radiation escapes from the atoms with an atomic number of 55, but only 10% escapes from atoms with atomic numbers less than twenty (6). The Auger effect is responsible for this difference in fluorescent yield. Normally when a K electron is knocked out of an atom, another electron will replace it, giving rise to one of the K series emission lines. The Auger effect occurs when the K emission radiation does not leave the atom but instead knocks out an L electron to generate an L series. The probability of the Auger effect occurring increases with a decrease in atomic number.

There are other factors besides the Auger effect which limit the applicability of X-ray fluorescence to the determination of the lower atomic number elements. One of these factors is the air absorption of the X-ray radiation as it travels through this medium. The weaker radiations of the lower atomic number elements are, of course, more easily absorbed. It is for this reason that a helium atmosphere or a vacuum path is necessary in order to determine the "light elements."

Background Radiation.—As in other spectroscopic methods of analysis, the line-to-background ratio is critical when considering the lower limits of detection of a method. Hence some mention should be made of the processes which produce the background radiation in X-ray fluorescence and therefore limit the range of detection. There are two scattering processes, coherent and Compton, which increase the background intensity.

Coherent scattering or unmodified scattering occurs when a photon undergoes an elastic collision with the sample. In this process the primary radiation is not absorbed nor does it cause fluorescence. It is merely reflected unchanged off the sample. Thus continuous background and weak characteristic primary radiation lines are always found superimposed on the fluorescent spectrum of a sample.

Compton or incoherent scattering occurs when an X-ray photon strikes an atom, especially the lower atomic number atoms such as oxygen, carbon, or hydrogen, and it is inelastically scattered, losing part of its energy. This loss of energy results in an increase in wavelength of the scattered X-ray photons. For a sample containing a high percentage of hydro-

carbons or other light elements, the Compton scattering will increase relative to the coherent scattering and may even be stronger than the coherent scattering.

Background scattering has a decided influence on the line-to-background ratio and therefore on the limit of detectability. The limit of detection of an element by X-ray fluorescence has been related to the background intensity by Birks (1). Birks states that the minimum composition detectable by X-ray fluorescence is that concentration that yields an intensity equal to three standard deviations of the background intensity. Thus to be detectable, a line must be at least three standard deviations above the background. Using this criterion, some typical orders of magnitude values for the minimum detectable limit, in parts per million, are shown in Table I.

METHODS OF ANALYSIS

Qualitative Analysis.—In order to determine which X-ray detectable elements are present in a sample, one merely has to scan through a wide 2 θ degree range recording the reflected intensities. That is, with the present-day commercial X-ray equipment it is possible to automatically scan and record, obtaining a plot of intensity *versus* 2 θ angle as shown in Fig. 10. From a chart recording of this kind, one obtains information as to what elements are

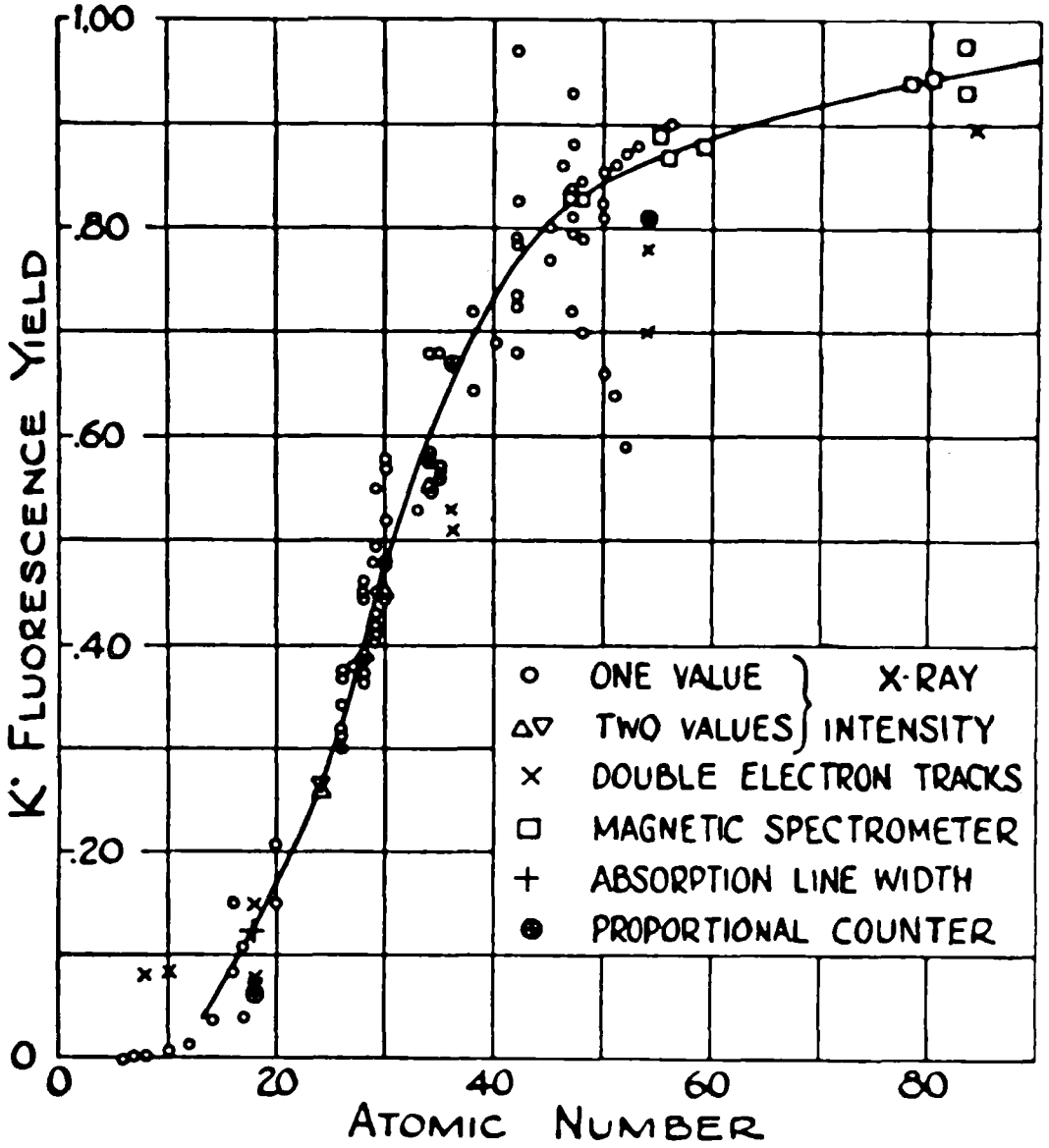

Fig. 9.—Fluorescent yield as a function of atomic number. [Reprinted with permission from Birks, "X-Ray Spectrochemical Analysis," Interscience, 1959.]

TABLE I.—MINIMUM DETECTABLE LIMIT

Nonconcentrated	Concentrated
10 p.p.m.	0.01 p.p.m.[a]
100 p.p.m. for light element in heavy matrix	
1 p.p.m. for medium element in light matrix	

[a] There is, of course, no absolute lower limit if large enough starting quantities are used. There are, however, practical limitations on both quantity and preparation time. The value listed represents a limit actually achieved in what is considered a practical analytical procedure (1).

present and their relative concentrations. It should be borne in mind that a great variety of sample forms can be analyzed both qualitatively and quantitatively by means of X-ray fluorescence (7). For example, analyses of rocks, powders, metal plates, wires and bars, briquets, solutions, films, evaporated residues, and massive solids are all possible with only slight if any instrumental modifications.

Quantitative Analysis—To quantitatively determine the concentration of a particular element in a sample, the goniometer must be positioned at a 2 θ angle peculiar to the element in question. Usually this 2 θ angle is the angle corresponding to the wavelength at which the $K\alpha$ first-order line of the sample element is reflected by the analyzing crystal. The counter is then allowed to accumulate either a fixed number of counts, the time for such a count being recorded, or to count for a fixed period of time. It is necessary to relate this intensity measurement to a per cent composition value for this element in the sample.

The relationship between measured intensity and elemental content of sample is controlled by what is known as the matrix effect. The matrix is everything which is present in the sample with the exception of the element of interest. Thus the matrix effect, which consists of both absorption and enhancement effects, is the effect which the elemental composition or matrix of the sample has upon the incident and diffracted radiation. Figure 11 is an illustration of the effect that the matrix has on the intensity-composition relationship. The matrix effect must be corrected for either by computation or physical methods. The computation methods are rather involved and are less popular than the physical methods.

The three principal physical methods are use of calibration standards, use of an internal standard, and the dilution technique.

In the calibration standard method, a series of standards is prepared with varying concentrations of the element in question but with a matrix identical to that of the sample. From this standard series a calibration curve of concentration *versus* intensity can be made. Thus, once the intensity from the unknown sample is obtained, the elemental concentration can be found by use of the calibration curve. In this method the matrix effect is eliminated by duplicating the sample matrix in the prepared standards.

When it is difficult or impossible to reproduce the matrix of the sample, one often resorts to the use of the internal standard. In this procedure the internal standard is added to the sample matrix and the prepared standard matrix. In this case the sample and standard matrix do not have to be identical. What actually is plotted in order to obtain the concentration of the element in the sample is the ratio of the internal standard intensity to the desired element intensity *versus* the desired element composition.

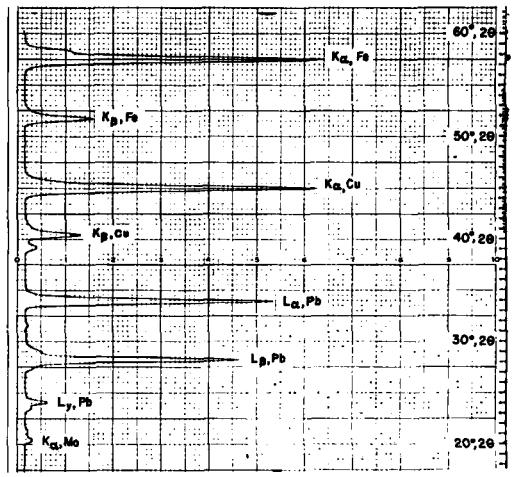

Fig. 10.—X-ray fluorescent chart recording of a sample consisting of copper, lead, and iron.

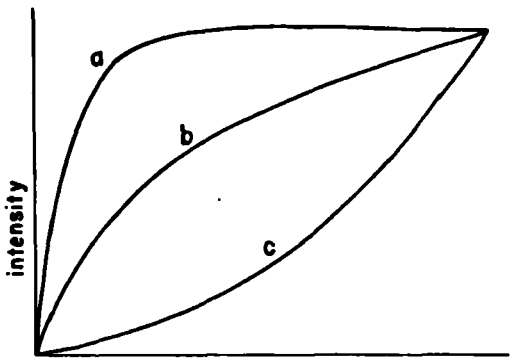

Fig. 11.—Variation of the intensity *vs.* composition curve with matrix effect. Curve *a*, a heavy element in a light-element matrix; in curve *b*, the absorption of the matrix is only slightly less than the self-absorption of the desired element; in curve *c*, the matrix absorption is slightly greater than the self-absorption of the desired element. [Reprinted with permission from Birks, "X-Ray Spectrochemical Analysis," Interscience, 1959.]

It is important that the internal standard chosen have an atomic number close to that of the element in question so that the absorption and enhancement effects of the matrix will be similar for the two elements.

The third physical method, the dilution technique, is simply a method whereby the samples' absorption and enhancement effects are reduced to a negligible amount by a physical dilution of the sample in a relatively transparent material. Materials often used for this purpose are water, organic solvents, starch, alumina, and borate glass.

APPLICATIONS

The use of X-ray fluorescent analysis in the pharmaceutical industry is certainly not extensive, and, consequently, there are not a great many published applications; however, the authors will supplement the published works of others with some of their own unpublished experiences in order to give the reader a full appreciation of the usefulness of X-ray fluorescence.

Qualitative Inspection.—X-ray spectroscopy is often utilized for the qualitative inspection of raw materials, intermediates, and final products. The reason for this is that a quick X-ray fluorescent scan will immediately reveal whether or not metal contamination has occurred or whether or not the concentration of a metal in a product has varied. Thus fluorescent scans are routinely taken of certain raw materials to assure the chemist that the same quality of material is being received.

Routine Quantitative Procedures.—There are several good examples of quantitative X-ray methods that have been adopted as routine control procedures. In almost all of these cases the analysis by any other method was either impossible or prohibitively time consuming.

R. B. Scott developed an X-ray method of analysis for the alkaloid hyoscine hydrobromide in a tablet formulation which contained one hundred times as much diphenhydramine hydrochloride (8). It was possible to analyze for halide conventionally, but the predominance of chloride was so great that the assay was meaningless for the small percentage of bromide. The X-ray fluorescent method using the $K\alpha$ line of bromine was found to be precise, convenient, fast, and unhampered by the presence of chloride.

The analysis of most of the minerals in vitamin-mineral supplement products is one of the most outstanding applications of X-ray fluorescence in this indusitry. Manganse, iron, potassium, zinc, calcume, and copper have been quantitatively assayed in a vitamin-mineral preparation by use of an internal standard technique (9). A wet chemical method of analysis of the minerals in such a product is at least ten times more time consuming than the X-ray method.

The arsenic content of arsenosobenzene, a coccidiostat used in veterinary food products is determined by an X-ray spectrographic method (9). The wet chemical method for arsenic in this product took 2 to 3 days to complete, whereas the X-ray method requires only 1 hour.

The analysis of merthiolate, a mercurial preservative, at the fifty parts per million level in pharmaceutical preparations is a relatively simple task if one assays for the mercury present by X-ray fluorescence (10).

The use of selenium dioxide and selenium dioxide in the presence of mercury as dehydrogenation catalysts in the chemical manufacture of certain steroids necessitated the analysis of selenium and mercury in the final product. Olson and Shell developed an X-ray fluorescent method of analysis for the determination of selenium and selenium and mercury in the presence of each other at the parts per million range (11, 12). This method has an accuracy of ± 1 p.p.m. in the range of 2 to 40 p.p.m. for both selenium and mercury.

Special Problems.—X-ray fluorescence has been quite useful as a tool in the solution of certain perplexing problems. The following are some examples of these problems.

A large pharmaceutical firm was confronted with the problem of a precipitate appearing in their diphtheria-tetanus toxoid preparation after the material had been packaged (8). An X-ray fluorescent scan of the precipitate revealed that zinc was present. This discovery led to an investigation of the rubber stoppers of the container since zinc is often used in rubber compounding. An X-ray examination of the rubber closures revealed zinc to be present. Obviously, the precipitation in the preparation was caused by zinc that was leached out of the rubber stoppers. The problem was quickly eliminated by changing closure specifications with respect to zinc content.

Another firm was faced with the problem of reporting to the Federal Food and Drug Administration the amount of ink present on an imprinted capsule (9). This problem was solved by an X-ray fluorescent determination of the titanium content in the ink itself and on the capsule. Calculations revealed that each capsule bore 13 mcg. of ink.

Trace Analysis.—Possibly one of the most dramatic applications of X-ray emission spec-

troscopy in the pharmaceutical industry has been in the determination of trace quantities of metallic elements either present as impurities or intentionally added in pharmaceutical formulations. X-ray emission spectroscopy acts as a a fingerprint of the formulation and has been used to positively identify the source of manufacture of the product. This technique, due to its high sensitivity, quantitative aspects, and nondestructive nature, has proven infallible to date.

Organic Analysis Through Inorganic Association.—As previously mentioned, those elements with an atomic number of 11 or below cannot be assayed by X-ray fluorescent methods because of the minimal fluorescent yield of these elements. Thus, the direct analysis of organic compounds by X-ray fluorescent methods is impossible at the present time. This is a great advantage when considering the analysis of a heavy element in an organic matrix, but it is also a serious limitation on a fine tool. Consequently, work has been done in this laboratory to illustrate the feasibility of determining organic substances indirectly through their association with an X-ray detectable element (13). Three different methods of associating an X-ray detectable element with an organic compound and assaying for this element have already been explored. They are the determination of phenolic and unsaturated compounds by bromination, the determination of 5-chloro-7-iodo-8-quinolinol by chelation, and the determination of ammonium acetate by salt formation. These methods provide specificity, selectivity, and sensitivity. Specificity and selectivity are obtained by the proper choice of the inorganic association reaction. Thus in many cases lengthy, time-consuming, error-introducing separation procedures may be eliminated. Such methods also have the potential of assaying, without isolation, microgram quantities in highly colored, many component solutions. This method should be quite useful in the analysis of drugs and metabolites in body fluids. In support of this statement, it might be mentioned that Natelson and Bender have already determined the calcium, chlorine, sulfur, and potassium content of a 20-μl. sample of serum by X-ray fluorescence (14, 15).

CONCLUDING REMARKS

Advantages and Disadvantages.—A consideration of the advantages and disadvantages of X-ray emission methods will emphasize the major points presented in the main body of this text.

The advantages of X-ray emission spectroscopy can be listed as follows:

1. Rapid.
2. Nondestructive.
3. Trace determinations possible.
4. Many sample forms are acceptable; *e.g.*, solutions, powders, intact tablets, *etc.*
5. Organic compounds do not interfere in the analysis of heavy atoms.
6. Equipment can easily and rather inexpensively be modified for diffraction use as well.
7. Once methods are developed, they can easily be performed by a technician.

The principal disadvantages are the initial cost of the equipment, which is \$15,000 to \$20,000, and the fact that direct analysis of organic compounds and elements with an atomic number less than 12 is not possible at the present time.

Future.—A number of uses for X-ray emission spectroscopy in pharmaceutical analysis have already been cited; however, as more investigators become involved in its use, one can be sure that many more applications will be uncovered.

As a matter of fact, it has already been shown that X-ray emission lends itself quite nicely to automation for the analysis of large numbers of samples as well as to the analysis of dynamic systems (1). It is highly probable that in the near future extensive use will be made of X-ray emission as an automatic analyzer in quality control laboratories. Also, greater use will be made of X-ray fluorescence in dynamic systems studies such as rate of solution, rate of precipitation, *etc.*

The vast amount of research that is now in progress on analyzing crystals, detectors, and X-ray tubes will someday make it possible to determine some, if not all, of the lower atomic number elements by X-ray emission methods.

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Resolution of Binary Mixtures by Partial Extraction and Specialized Spectrophotometric Techniques

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One of the most common procedures in analytical chemistry is the separation of two substances by solvent–solvent extraction. Complete separation of the two ingredients in the binary mixture is, however, unnecessary for quantitative assay purposes. Simultaneous analysis of the ingredients is possible by using the relationship that exists between measured partition coefficients and the relative concentration of one of the substances in the binary mixture. The method is based on the distribution of the pure substances between two immiscible solvents and on specialized spectrophotometric techniques.

ONE OF THE FIRST steps in the analysis of a pharmaceutical is the separation of one or more of the ingredients from the bulk form of the dosage unit. This is most often carried out by the solvent–solvent extraction process based on the distribution law. Analytical conditions are usually so fixed that at the completion of the extraction process only one of the ingredients is present in one of the phases of the two-phase system. If these conditions are altered in such a way that both ingredients in a binary system are present in both phases, then a relationship is established between the measured partition coefficient of the mixture and the relative concentration of one of the ingredients in such a mixture. Furthermore, simultaneous analysis does not require the total separation of the ingredients but only partial separation under controlled conditions. This technique is thus analogous to the absorbancy ratio method of analysis described in previous publications (1, 2). The principles of controlled partial extraction are outlined in the literature (3, 4) but the procedures described therein differ from those in this paper in the types of measurements used to evaluate total concentrations and in the mathematical manipulations required in the analysis.

It is not the intention of the authors to discuss or elaborate on the subject of partition coefficients. The fundamentals inherent in the distribution law and the pitfalls associated with the determination of partition coefficients are set forth in textbooks on physical pharmacy (5) or physical chemistry (6). Factors such as temperature, phase volumes, association, dissociation, or chemical reaction of the component with one or both of the solvents must be considered in any investigation involving distribution between two immiscible solvents.

The spectrophotometric terminology and symbology used in this paper shall be that suggested by the National Bureau of Standards (7). Thus, absorbancy (A_s) is equal to the product of the absorbancy index (a_s), the cell length (b), and the concentration (c).

THEORY

When component X is distributed between two immiscible phases, the equilibrium state may be expressed mathematically in the following way

$$K_x = \frac{C_{xu}}{C_{xl}} \qquad \text{(Eq. 1)}$$

K_x, the partition coefficient, is thus equal to the ratio of the concentration of the X component in the upper layer (C_{xu}) to that in the lower layer (C_{xl}). Similarly, the partition coefficient for component Y, K_y, distributed between the same two solvents under similar experimental conditions, is defined as

$$K_y = \frac{C_{yu}}{C_{yl}} \qquad \text{(Eq. 2)}$$

The concentrations of Y in the upper and lower phases are equal to C_{yu} and C_{yl}, respectively. If the distribution of X is not influenced by Y and vice versa, the partition coefficient for the mixture (K_m) containing X and Y may be defined as

$$K_m = \frac{C_{xu} + C_{yu}}{C_{xl} + C_{yl}} \qquad \text{(Eq. 3)}$$

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However, the sum of the concentrations in the two layers is equal to the total concentration of X, C_x, and Y, C_y, in the binary mixture. The concentration of X in the upper layer is thus equal to $C_x - C_{xl}$. This latter value is now substituted for C_{xu} in Eq. 1

$$K_x = \frac{C_x - C_{xl}}{C_{xl}} \qquad \text{(Eq. 4)}$$

Eq. 4 is rearranged

$$C_{xl} = \frac{C_x}{K_x + 1} \qquad \text{(Eq. 5)}$$

Using similar mathematical manipulations, Eq. 1 is defined in terms of C_x and C_{xu}

$$C_{xu} = \frac{K_x C_x}{K_x + 1} \qquad \text{(Eq. 6)}$$

In exactly the same way as shown in Eqs. 5 and 6, C_{yu} and C_{yl} are defined in terms of C_y. All of these quantities are substituted into Eq. 3

$$K_m = \frac{\frac{K_x C_x}{K_x + 1} + \frac{K_y C_y}{K_y + 1}}{\frac{C_x}{K_x + 1} + \frac{C_y}{K_y + 1}} \qquad \text{(Eq. 7)}$$

K_1 is equal to $K_x/(K_x+1)$, K_2 to $K_y/(K_y+1)$, K_3 to $1/(K_x+1)$, and K_4 to $1/(K_y+1)$. Each term in Eq. 7 is divided by $C_x + C_y$. $C_x/(C_x + C_y)$ is now equal to F_x, the fraction of X component in the binary mixture, and $C_y/(C_x + C_y)$ is equal to F_y, the fraction of Y component in the binary mixture

$$K_m = \frac{K_1 F_x + K_2 F_y}{K_3 F_x + K_4 F_y} \qquad \text{(Eq. 8)}$$

However, the sum of F_x and F_y is equal to 1. F_y is, therefore, equal to $1 - F_x$. This latter quantity is substituted for F_y above and the equation is rearranged

$$K_m = \frac{F_x (K_1 - K_2) + K_2}{F_x (K_3 - K_4) + K_4} \qquad \text{(Eq. 9)}$$

A plot of K_m *vs.* the fraction of X in the binary mixture is thus a curve, the extremities of which are equal to the partition coefficients of X, K_x, and Y, K_y. To use this equation K_m, K_x, and K_y must be assigned numerical values during the course of an analysis. This can be conveniently carried out by using the appropriate spectrophotometric techniques.

The determination of partition coefficients by carrying out absorbancy measurements on the two phases of the system has been previously described in the literature (8). Such measurements are usually carried out at those wavelengths at which the pure substance exhibits maximum absorption. Such a technique cannot be used to determine K_m since the phases contain two substances. The total concentration in any one phase can, however, be determined by carrying out absorbancy measurements at an isoabsorptive point, that is, that wavelength at which the two substances have the same absorbancy index value. Methods for locating such a point have been described in a previous publication (2).

At the isoabsorptive point, the following equations are valid

$$C_{xu} + C_{yu} = A_u/a \qquad \text{(Eq. 10)}$$

$$C_{xl} + C_{yl} = A_l/a \qquad \text{(Eq. 11)}$$

The absorbancy index value at the isoabsorptive point is equal to a. A_u is equal to the *total* absorbancy at the isoabsorptive point in the upper phase and A_l is equal to the *total* absorbancy at the isoabsorptive point in the lower phase.

The right-hand members of Eqs. 10 and 11 may now be substituted for K_m in Eq. 3

$$K_m = \frac{A_u/a}{A_l/a} = \frac{A_v}{A_l} \qquad \text{(Eq. 12)}$$

Therefore, a plot of A_u/A_l *vs.* F_x, the fraction of X in the binary mixture, is a curve, the extremities of which are equal to K_x and K_y. A binary mixture may now be analyzed by distributing the pure substances and the binary mixture between two immiscible solvents under controlled conditions.

Eq. 9 can yield only relative quantities but absolute analysis can be carried out by utilizing the above mathematical principles in a slightly different form. From Eqs. 1 and 2, C_{xu} is equal to $K_x C_{xl}$ and C_{yu} to $K_y C_{yl}$. The appropriate terms are substituted for C_{xu} and C_{yu} in Eq. 3

$$K_m = \frac{K_x C_{xl} + K_y C_{yl}}{C_{xl} + C_{yl}} \qquad \text{(Eq. 13)}$$

K_m is, however, equal to A_u/A_l and $C_{xl} + C_{yl}$ to A_l/a. C_{yl} is thus equal to $A_l/a - C_{xl}$. These quantities are substituted into Eq. 13

$$\frac{A_u}{A_l} = \frac{K_x C_{xl} + K_y (A_l/a - C_{xl})}{C_{xl} + (A_l/a - C_{xl})} \qquad \text{(Eq. 14)}$$

The equation is rearranged, each term is multiplied by a and divided by A_l

$$\frac{A_u}{A_l} = \frac{C_{xl}}{A_l}(aK_x - aK_y) + K_y \qquad \text{(Eq. 15)}$$

However, the total concentration of X, C_x, in the binary mixture is equal to $C_{xu} + C_{xl}$. Therefore, C_{xu} is equal to $C_x - C_{xl}$. This latter quantity is substituted into Eq. 1

$$C_{xl} = \frac{C_x}{K_x + 1} \qquad \text{(Eq. 16)}$$

The right-hand member of the above equation is substituted for C_{xl} in Eq. 15

$$\frac{A_u}{A_l} = \frac{C_x}{A_l} \cdot \frac{aK_x - aK_y}{K_x + 1} + K_y \qquad \text{(Eq. 17)}$$

A plot of A_u/A_l *vs.* C_x/A_l results in a straight line with a slope value of

$$\frac{aK_x - aK_y}{K_x + 1}$$

and an intercept value of K_y. Eq. 17 may now be used to determine the quantity of X component in the binary mixture.

An acetophenetidin-caffeine system was investigated to illustrate the method described herein.

EXPERIMENTAL

Apparatus.—(*a*) Beckman model DU spectro-

photometer; (*b*) Eberbach automatic shaker; (*c*) Thomas-Hoover capillary melting point apparatus.

Reagents and Solutions.—(*a*) Acetophenetidin, recrystallized from ethanol, the sample melted between 134.5 and 135.5°; (*b*) caffeine, recrystallized from ethanol, the melting point of the substance was 235.0–236.0°; (*c*) water, saturated with *n*-butanol: add 100 ml. of reagent grade *n*-butanol to 250 ml. of water, shake well, and allow to stand at room temperature for 30 min., filter the aqueous layer through Whatman No. 1 filter paper; (*d*) *n*-butanol, saturated with water: add 100 ml. of water to 250 ml. of reagent grade *n*-butanol, shake well, and allow to stand at room temperature for 30 min., filter the butanol layer through Watman No. 1 filter paper.

Spectral Characteristics of Acetophenetidin and Caffeine.—In water, acetophenetidin absorbs ultraviolet radiant energy most strongly at 245 mμ. Caffeine exhibits maximum absorption at 273 mμ. An isoabsorptive point occurs at 261.5 mμ. Methods for locating isoabsorptive points were described in a previous publication (2).

Solutions were prepared by dissolving the substance in 25.00 ml. of ethanol and diluting to 1 L. with water. Aliquots of such stock solutions were diluted with water to give final concentrations of approximately 8–10 mg. of substance per liter of solution.

Determination of Partition Coefficients.—To a weighed sample of either acetophenetidin or caffeine, add 25.00 ml. of *n*-butanol and 50.00 ml. of water. (References to *n*-butanol and water in the text of this paper imply the solutions described in the second part of this section.) Shake for 1 hour at room temperature. Allow the layers to separate, withdraw suitable aliquots, dilute to 1 L. with water, and determine absorbancy values. The *total* absorbancy in the butanol phase divided by the *total* absorbancy in the aqueous phase is equal to the partition coefficient for the substance.

The partition coefficient for caffeine was found to be 0.83 ±0.01. This value represents six determinations on samples ranging in weight from 0.1990 to 0.2136 Gm. Two-milliliter aliquots of the butanol phase and 4.00-ml. aliquots of the aqueous phase were taken for the determinations. The partition coefficient for acetophenetidin was equal to 16.40 ±0.09. Six samples, ranging in weight from 0.1274 to 0.2078 Gm., were processed in the manner described above. Aliquots of 1.00-ml. (butanol phase) and 25.00-ml. (aqueous phase) were used in the determinations.

Relative Analysis of Mixtures Containing Acetophenetidin and Caffeine.—A 200-mg. sample of the mixture was accurately weighed and transferred to a 125-ml. Erlenmeyer flask. Twenty-five milliliters of butanol and 50.00 ml. of water were added to the flask. The flask was continuously agitated for 1 hour at room temperature. Suitable aliquots were then taken for absorbancy measurements. For commercial preparations containing $2^1/_2$ grains of acetophenetidin and $^1/_2$ grain of caffeine, 2.00 ml. of the butanol phase and 20.00 ml. of the aqueous phase, diluted to 1 L., will give suitable absorbancy readings. All absorbancies were determined at 261.5 mμ. The total absorbancy in the butanol phase divided by the total absorbancy in the aqueous phase is equal to K_m. Eq. 9 may now be used in the analysis of the binary mixture. The numerical form of this equation is

$$K_m = \frac{0.489\ F_x + 0.545}{-0.489\ F_x + 0.546}$$

F_x is equal to the relative concentration of acetophenetidin in the binary mixture. The relative contration of caffeine in the mixture may be determined by subtracting F_x from 1, or by using an equation similar to that above but defined in terms of caffeine. The results of the analysis of 10 mixtures of acetophenetidin and caffeine are reported in Table I.

Table I.—Results of the Analysis of Mixtures Containing Acetophenetidin and Caffeine

Mixture	Acetophenetidin Present, %	Acetophenetidin Found, %	Caffeine Present, %	Caffeine Found, %
1	90.9	90.9	9.1	9.1
2	87.7	87.7	12.3	12.3
3	85.0	84.9	15.0	15.1
4	80.0	80.2	20.0	19.8
5	78.9	79.4	21.1	20.6
6	75.1	75.5	24.9	24.5
7	69.4	68.9	30.6	31.1
8	60.0	60.3	40.0	39.7
9	50.7	51.2	49.3	48.8
10	39.4	40.4	60.6	59.6

Absolute Determination of Acetophenetidin in Mixtures.—The analytical manipulations are identical to those described above. Eq. 17 must be put in its numerical form in order to complete the analysis. This was done by preparing 10 synthetic mixtures containing known quantities of acetophenetidin and caffeine, subjecting the mixtures to the above procedure, and substituting the appropriate numbers for A_u and A_l in Eq. 17. The data were plotted as shown in Fig. 1 and subjected to the method of least squares (9). The numerical form of Eq. 17 is

$$K_m = A_u/A_l = \frac{30.99\ C_p}{A_l} + 0.88$$

In the analysis of unknown mixtures, the total

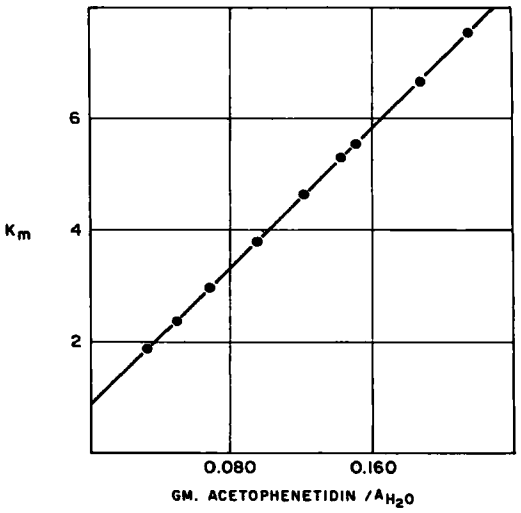

Fig. 1.—Calibration curve for the determination of acetophenetidin in the presence of caffeine.

absorbancy in the butanol phase (A_u) and the total absorbancy in the aqueous phase (A_l) are knowns. C_p, the concentration of acetophenetidin in the sample taken in the analysis, can then be calculated from the above equation. The results of the analysis of acetophenetidin in synthetic mixtures are shown in Table II.

TABLE II.—RECOVERY OF ACETOPHENETIDIN FROM MIXTURES CONTAINING ACETYLSALICYLIC ACID, ACETOPHENETIDIN, AND CAFFEINE

Acetophenetidin Present, Gm.	Acetophenetidin Recovered, Gm.	Recovery, Per Cent
0.1556	0.1531	98.4
0.1497	0.1512	101.0
0.1600	0.1597	99.8
0.1606	0.1606	100.0
0.1607	0.1609	100.1
0.1642	0.1641	99.9
0.1711	0.1732	101.2
0.1619	0.1603	99.0
0.1618	0.1617	99.9
0.1610	0.1603	99.6
Average Recovery		99.9
Standard Deviation		±0.8

Determination of Acetophenetidin in APC Tablets.—Add 50 ml. of chloroform to an amount of sample equivalent to one tablet. Shake to dissolve as much of the powdered material as possible and then add 10 ml. of 5% sodium bicarbonate solution. Shake, draw off the chloroform layer, and wash with 15 ml. of distilled water. Extract the combined bicarbonate and wash solutions with 2 × 15-ml. portions of chloroform. Wash the chloroform with 10 ml. of water, combine the chloroform extracts, filter through a pledget of cotton wool, wash the cotton wool with a few ml. of chloroform, and evaporate the organic solvent. To the residue, add water and butanol and continue as indicated above. The results of the analysis of a number of commercial preparations are shown in Table III.

TABLE. III.—RESULTS OF THE ANALYSIS OF COMMERCIAL PREPARATIONS CONTAINING ACETYLSALICYLIC ACID, ACETOPHENETIDIN, AND CAFFEINE

Product	Acetophenetidin per Tablet, mg. Label Claim	Found
A	129.6	125.8
		127.1
B	162.0	157.7
		154.8
C	162.0	156.0
		157.5
D	162.0	162.5
		160.5
E	162.0	166.6
		165.4

DISCUSSION

This method of analysis is based on a single but controlled extraction and on specialized spectrophotometric measurements. Although this confers a degree of specificity to the technique, it also limits its applicability to those binary mixtures which meet the conditions implied by the mathematical derivations. These limitations are mostly those associated with the distribution law and with the spectrophotometric measurements required by the assay.

Eq. 9 yields relative concentrations but places no limitations on the procedures used to determine K_m, K_x, and K_y. Eq. 12, on the other hand, specifies absorbancy measurements at an isoabsorptive point. Consequently, the two components in the binary mixture must characteristically absorb ultraviolet radiant energy and, more important, must show the presence of an isoabsorptive point when the absorbancy index–wavelength curves are superimposed. The exact nature of the spectrophotometric curves is not too important but if the spectrophotometric differences between the two substances are optimal, then some other method of analysis may prove to be faster and more accurate. This method of analysis is, therefore, applicable to those substances which have essentially similar spectral characteristics.

Eq. 9 implies that a plot of K_m *vs.* the relative concentration of one of the substances in the binary mixture should result in a curve. This was found to be experimentally true and such a curve is shown in Fig. 2. This curve is based on the data obtained by subjecting known mixtures of acetophenetidin and caffeine to the procedure described in the previous section. An examination of this figure indicates one of the limitations of this method of analysis. Even though the partition coefficients of the pure substances differ significantly, the rate of change of K_m with relative concentration is small in the 0 to 60% acetophenetidin region. Mixtures having concentrations defined by the above limits will not lend themselves to accurate analysis and consequently this method becomes inoperative. The reason for choosing the binary mixture containing acetophenetidin and caffeine to illustrate the applicability of this technique is thus almost self-evident.

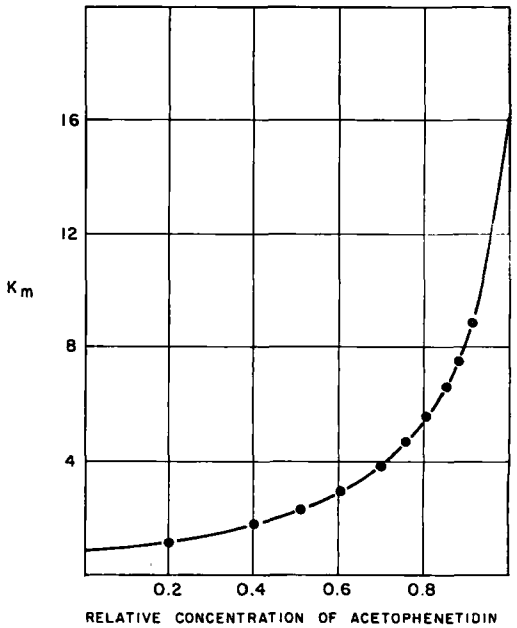

Fig. 2.—Partition curve for acetophenetidin and caffeine.

Commercial preparations usually contain 85 parts of acetophenetidin and 15 parts of caffeine. Fig. 2 shows that mixtures containing 85% acetophenetidin can be analyzed with good accuracy because the rate of change of K_m with respect to relative concentration is now considerably greater than that for lesser concentrations. A curve such as that shown in Fig. 2 must, therefore, be constructed in order to assess the relative accuracy of the method. If the particular mixture under investigation does not fall in an optimal range then some other method of analysis must be used.

The equations derived in the previous section imply that no spectrophotometric constants or partition values are required prior to analysis. Necessary data can be accumulated by analyzing synthetic mixtures or mixtures in which the absolute concentration of only one of the two substances is known. For example, the calibration curve illustrated in Fig. 1 can be constructed from data obtained by subjecting weighed quantities of acetophenetidin and unknown quantities of caffeine to the distribution process described in the previous section. The direct approach is to determine K_x, K_y, and a separately and then substitute the appropriate values into Eq. 17. This was done for the mixture investigated herein. When the determined partition and absorbancy index values at 261.5 mμ were substituted into Eq. 17, certain discrepancies between the numerical form so calculated and that reported in the previous section were observed. The slope value was found to be 31.59 and the intercept value 0.83. The latter value differs from that reported in the previous section by approximately 2%, a not unacceptable error. The discrepancy between intercept values is somewhat greater (that is, 0.83 as compared to 0.88). This discrepancy is not too significant provided the relative concentration of acetophenetidin is 60% or more and is probably due to the factors cited in the previous paragraph. It is preferable, therefore, to obtain the numerical form of Eq. 17 by the method of least squares from data obtained by analyzing actual mixtures of the two substances. This not only avoids a tedious, direct determination of constants but also serves as a check on the validity of Eq. 17.

The general shape of Fig. 2 is governed by the partition coefficient values of the two components and these, in turn, are governed by the volumes of the two phases used in the first step of the determination. By changing the ratio of one solvent to the other, the partition coefficient values will change and hence alter the accuracy of the method. For example, initial investigations on the acetophenetidin-caffeine mixture were carried out by using 50.00 ml. of *n*-butanol and 50.00 ml. of water. The results obtained under such circumstances were not as satisfactory as those shown in Table 1. The volumes were then changed to 25.00 ml. of *n*-butanol and 50.00 ml. of water in order to provide a more favorable distribution of the two components between the two immiscible solvents. This might be considered as one of the advantages of the method, that is, by altering volumes, the conditions of assay and hence the accuracy can be easily changed. On the other hand, it is somewhat difficult to determine optimal conditions for the most accurate analysis of the binary mixture. As a general rule, conditions should be so fixed that there is a maximal difference between the partition coefficient values of the pure substances. Substance solubility and ease of handling of smaller quantities of solvent will limit the applicability of this rule.

Any two immiscible solvents can be used in this type of analysis provided the two components of the mixture show complete solubility when subjected to the distribution procedure. The limitation here is the temperature coefficients of the particular solvents used in the analysis. All the determinations described in this paper were carried out at room temperature and because this temperature varied only slightly from day to day, the accuracy of the method was found to be excellent. This, of course, may not always occur (and, in fact, certain solvents are more susceptible to slight varations in temperature) and, consequently, a more accurate control of temperature would be necessary. This in itself is a disadvantage and implies a criticism of the method. There is yet a second factor to consider. Partition coefficient values depend not only on the volumes of the two phases but also on the inherent characteristics of the solvents. Although this was not investigated, changes in pH, for example, will alter the solubility of a particular substance in one of the phases and thus change the numerical value of the partition coefficient.

If the total weight of the mixture being analyzed is known, then relative concentrations can be easily converted to absolute quantities. Even though most methods of analysis begin with a weighing, the sample often contains substances other than those being analyzed. There is thus no way of determining the total weight of the active ingredients unless some secondary step is introduced into the method. The simplest approach is to carry out a complete isolation of the two components, determine the total weight, and then subject the mixture to the procedure described in the previous section. Another possible approach is to determine the absorbancy index value at the isoabsorptive point. The total absorbancy in both phases divided by the absorbancy index value is equal to total quantity of mixture present. The absolute concentrations of the two components can then be calculated. Both approaches suffer from a number of inherent errors. The gravimetric approach is tedious and does not guarantee that the isolated material being weighed is actually composed of only two substances. The spectrophotometric approach involves the determination of absorbancy index values at an isoabsorptive point. While this is feasible (2), the necessary manipulations are sufficiently involved to make this approach undesirable.

Eq. 17 provides an alternative approach. Since this is the equation of a straight line, it can be used in its numerical form, a form that can be easily calculated from data obtained on a restricted number of synthetic mixtures. Concentrations are now in absolute terms and are more meaningful when comparing the analytical result with the quantity claimed on a label or manufacturing ticket.

This method of analysis has an accuracy of approximately one per cent under ideal conditions. If secondary steps are introduced into the procedure in order to isolate the two substances being analyzed, the precision and accuracy of the method will be somewhat less than that shown in Tables I and II. There is no indication, however, that such

errors become excessive since the agreement between duplicate analyses of commercial preparation containing acetophenetidin is generally satisfactory.

CONCLUSION

Eq. 9 is fundamental to this general type of analysis. All the basic advantages and disadvantages associated with this method are found therein. It is important, therefore, to prepare a plot of K_m *vs.* relative concentration before attempting an analysis based on controlled partial separation. The importance of this is evident from Fig. 2. If such a curve shows that a simultaneous analysis of the components of a particular binary mixture is possible, then the method commends itself to the analyst. This method is particularly useful in those cases where the usual spectrophotometric methods of simultaneous analysis are not possible because of the similarity of the spectrophotometric curves of the components and where the usual solvent–solvent extraction techniques are not capable of completely separating the two components. Table I shows that the method is accurate and precise. The method, therefore, provides another approach to the basic problem of the analysis of complex pharmaceuticals.

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Investigation of the Sedimentation Behavior of Dispersions

By AMY MOORE† and A. P. LEMBERGER

Using a modification of the method of Greiner and Vold, suspension isotherms were obtained for dispersions of zinc oxide, calcium carbonate, and bismuth subcarbonate in sodium lauryl sulfate, dioctyl sodium sulfosuccinate and sodium-salts of polymerized alkylnaphthalenesulfonic acids (Daxad 11) solutions. For zinc oxide and bismuth subcarbonate systems maximum suspendability was reached at surfactant concentrations somewhat beyond the critical micelle concentration and extended over a relatively short concentration range. In the case of calcium carbonate, limited suspendability was observed with all three surfactants. Previous workers attribute the reduction in suspendability at higher surfactant concentrations to a reduction in zeta potential, thus permitting increased aggregation. Suspension isotherms obtained at constant and varying ionic strengths of sodium chloride indicate that aggregation is facilitated by the presence of excess surfactant through some additional mechanism.

THE SEDIMENTATION behavior exhibited by a dispersion has long been recognized as being of interest and importance pharmaceutically. Separation of phases in a polyphase pharmaceutical product can result in failure to provide uniform doses of the drug or drugs suspended.

One phenomenon which may occur and contribute to increased rates of sedimentation in these dispersions is aggregation (1). Thus, a number of investigations of sedimentation or creaming rates have been made and aggregation behavior deduced in a qualitative or semiquantitative fashion (1–5).

Further interest in the phenomenon of aggregation arises from the observation that in addition to the effect on physical stability, aggregation may also contribute to the flow properties exhibited by emulsions and suspensions (6). Recently, W. I. Higuchi, Okada, and Lemberger (7) reported on the reversible aggregation-deaggregation of hexadecane-in-water emulsions containing dioctyl sodium sulfosuccinate (AOT). In this study a procedure involving rapid counting and sizing of droplets as a function of time was employed, thus permitting direct determination of aggregation in the system.

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errors become excessive since the agreement between duplicate analyses of commercial preparation containing acetophenetidin is generally satisfactory.

CONCLUSION

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One phenomenon which may occur and contribute to increased rates of sedimentation in these dispersions is aggregation (1). Thus, a number of investigations of sedimentation or creaming rates have been made and aggregation behavior deduced in a qualitative or semiquantitative fashion (1–5).

Further interest in the phenomenon of aggregation arises from the observation that in addition to the effect on physical stability, aggregation may also contribute to the flow properties exhibited by emulsions and suspensions (6). Recently, W. I. Higuchi, Okada, and Lemberger (7) reported on the reversible aggregation-deaggregation of hexadecane-in-water emulsions containing dioctyl sodium sulfosuccinate (AOT). In this study a procedure involving rapid counting and sizing of droplets as a function of time was employed, thus permitting direct determination of aggregation in the system.

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Sedimentation studies in which ionic surface-active agents have been employed reveal that in a given system, suspendability of the dispersed phase passes through a maximum at a given concentration of surface-active agent something in excess of its critical micelle concentration (2, 3, 5, 8). Increasing suspendability to the maximum is generally accepted as being due to selective adsorption of the anion (2, 3, 5, 8, 9). As the anion is adsorbed, the zeta potential of the particles increases which leads to deflocculation of aggregates. As a result of deflocculation, effective particle size is decreased and the rate of sedimentation under gravitational effects is reduced.

Two possible explanations for the observed decrease in suspendability beyond the maximum have been offered. Greiner and Vold (2) and Vold and Konecny (3) propose that the decrease in suspendability at higher concentrations of surface-active agent results from a decrease in zeta potential caused by the increased concentration of counter ions. However, Doscher, in his sedimentation studies (5), showed that sedimentation rates increased markedly at a concentration in excess of the critical micelle concentrations, while zeta potential decreased only slightly. Both he and Cockbain, reporting on the creaming of emulsions systems (1), suggest that adsorption of polymolecular aggregates occurs at higher concentrations, and that interaction of hydrophobic surfaces results in bridging between particles leading to aggregation. Higuchi, Okada, and Lemberger (7) recognized that both London dispersion forces, rendered more effective by decreased zeta potential, and film to film interactions may be important in aggregation phenomena. It is the purpose of this study to lend further support to this position.

EXPERIMENTAL

Materials.—The insoluble powders employed were zinc oxide U.S.P., calcium carbonate, reagent grade, and bismuth subcarbonate U.S.P. (light powder). These particular powders were chosen because of their widespread use in pharmaceutical suspensions.

The anionic surfactants used were sodium lauryl sulfate (NaLS), Aerosol OT (100% pure sodium dioctyl sulfosuccinate from American Cynamid), and Daxad 11 (sodium salts of polymerized alkyl naphthalene sulfonic acids from Dewey and Almy). The AOT and Daxad 11 were used without alteration. The NaLS was recrystallized twice with hot 95 per cent ethanol before use.

Sodium chloride U.S.P. was used in the experiments concerning electrolytes.

The distilled water used in the dispersions was decarbonated by vigorous boiling for fifteen minutes and cooling in a flask stoppered with a soda-lime tube apparatus.

Procedure.—The procedure employed was a modification of the method outlined by Greiner and Vold (2). Surfactant solutions of the desired concentrations were prepared in 250-ml. quantities at specific time intervals before use (3 hours for the AOT solutions and 24 hours for the Daxad 11 and NaLS solutions). The necessity for using solutions of uniform age was demonstrated by Greiner and Vold in their studies of the suspending powers of aged AOT solutions.

A small portion of this solution and 2 Gm. of the insoluble powder were placed in a ground-glass homogenizer and levigated vigorously for 2 minutes. The concentrated suspension was then transferred quantitatively to a graduated 12-in. test tube, using portions of the remaining surfactant solution. All test tubes were calibrated at the 50-ml. and 250-ml. levels. Both the diameter (3.7 cm.) and the distance between calibrations (23 cm.) were identical in every case. The liquid was brought up to volume (250 ml.) with the remaining surfactant solution and the tube clamped on the stirring apparatus. This consisted of an elongated glass spiral terminated with a soft rubber propeller fitted onto a small reduction motor. The suspension was stirred for 18 minutes, effecting a total stirring time of 20 minutes. The tube was then removed from the stirrer, stoppered, inverted slowly by hand several times, clamped in a vertical position, and allowed to stand undisturbed for a 2-hour period. At the end of this time the liquid was withdrawn to the 50-ml. mark, and assayed for the amount of powder which had remained suspended. A special J-tipped siphon and a slow withdrawal rate were employed to minimize the disturbance of the sediment.

The zinc oxide suspensions were assayed according to a modified U.S.P. XVI procedure in which the zinc oxide was dissolved in an excess of 1.0 *N* sulfuric acid in the presence of ammonium chloride, and the resulting solution back-titrated with 1.0 *N* sodium hydroxide.

The 1932 British Pharmacopoeia assay method was employed for calcium carbonate. The calcium carbonate was dissolved in an excess of 1.0 *N* hydrochloric acid, and back-titrated with 1.0 *N* sodium hydroxide. Methyl orange test solution (U.S.P. XVI) was used as an indicator in both of the above cases.

A gravimetric procedure was used for bismuth subcarbonate, whereby the 200 ml. of suspension were centrifuged approximately 20 minutes, or until the supernatant liquid was clear. The liquid was decanted, and the residue was transferred quantitatively to a tared crucible and dried to constant weight.

In the experiments demonstrating the influence of electrolytes on the suspending ability of the surfactants, the salt was dissolved in the surfactant solution before levigation was begun.

Suspension isotherms were obtained by plotting the milligrams per milliliter of insoluble powder remaining suspended after the 2 hours *vs.* the concentration of surfactant solution used.

It has been established that the effects of stirring time (10) and precontact[1] time (2) are critical factors in sedimentation analysis, and, therefore, were carefully controlled. Inasmuch as the suspending power

[1] Precontact time is the length of time which the powder and surfactant are allowed to remain in contact before the actual sedimentation period begins (2).

of detergents is generally independent of temperature (11), no effort was made to maintain a constant temperature experimental environment; all work was done at room temperature.

RESULTS AND DISCUSSION

The suspension isotherms obtained for the various insoluble powder–anionic surface-active agent combinations employed are given in Figs. 1–5. In accordance with results of previous workers, a maximum suspendability which extended over a relatively short concentration range was observed at a point somewhat beyond the critical micelle concentration of the surfactant.

An exception to the normal pattern was observed in the case of calcium carbonate. With all three anionic surface-active agents employed in this study only a slight increase in suspendability was noted

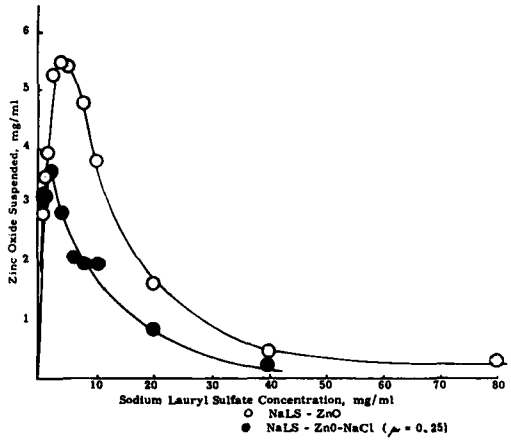

Fig. 1.—Suspension isotherms for zinc oxide–NaLS systems.

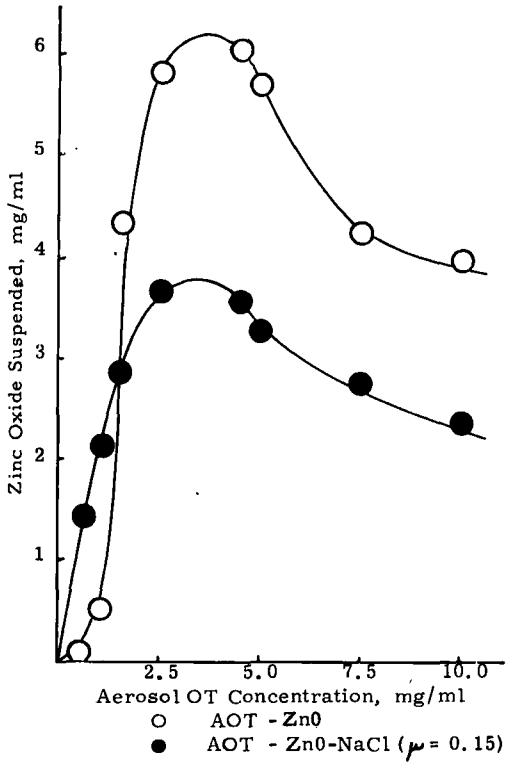

Fig. 2.—Suspension isotherms for zinc oxide–AOT systems.

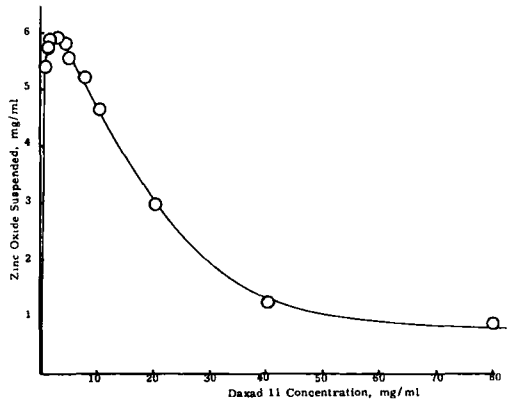

Fig. 3.—Suspension isotherm for zinc oxide–Daxad 11 system.

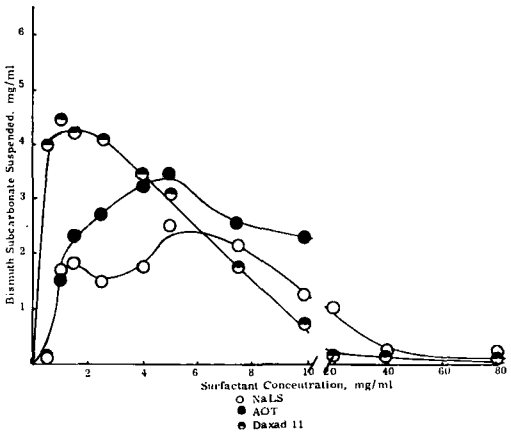

Fig. 4.—Suspension isotherms for bismuth subcarbonate with various anionic surface active agents.

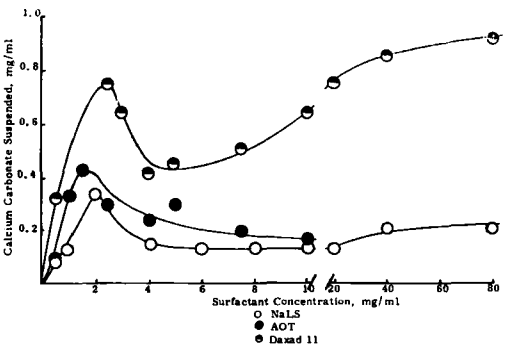

Fig. 5.—Suspension isotherms for calcium carbonate with various anionic surface-active agents.

in the initial portion of the suspension isotherm. With both Daxad 11 and NaLS, whose greater water solubility permitted determination of suspendability at higher concentrations, suspendability was observed to increase once again in the higher concentration range. Qualitative evidence for a high degree of aggregation in these systems which might in part account for their low order of suspendability is furnished by the observation that calcium carbonate sediments were more loosely packed than the other powder sediments.

A further objective of this study was to show experimentally that the zeta potential of the particles exerts a significant influence on the sedimentation characteristics of a dispersion. Since zeta potential is reduced or lost in the presence of high concentrations of electrolyte, suspension isotherms were obtained for zinc oxide–NaLS and zinc oxide–AOT systems in the presence of swamping electrolyte. For the zinc oxide–NaLS system, ionic strength was maintained at 0.25 and for the AOT system, at 0.15 with sodium chloride. For calculation of ionic strength, 100% dissociation of the surface-active agent was assumed. Although at higher concentrations the ionic strength would be somewhat less, it was felt that electrolyte concentration was sufficient to assure flooding of the system. Results of these experiments are given in Figs. 1 and 2.

In both instances maximum suspendability of the zinc oxide was significantly decreased indicating that zeta potential plays an important role in the suspendability of insoluble powders by contributing to the formation of a deaggregated system. It seems reasonable to expect the appearance of maxima in the suspension isotherms even though zeta potential is minimal because the NaLS and AOT could act as weakly ionic or nonionic surface-active agents in these systems. For the zinc oxide-NaLS system, the position of the maximum was shifted to a slightly lower concentration of NaLS and the width of the peak reduced. A possible explanation of this observation would be the greater ionic strength and/or an increased sensitivity to the presence of electrolyte in the case of NaLS.

Also of interest in suspendability behavior is the decrease observed beyond the maximum. At higherconcentrations of anionic surfactant, the concentr ation of counter ions increases and results in a decreased zeta potential. If the aggregation responsible for the increased sedimentation rates observed in this portion of the suspension isotherm is entirely due to London dispersion forces, rendered more effective because of the closer approach of particles under a decreased zeta potential, any source of counter ions should produce equivalent behavior in the system. For this reason suspension isotherms were obtained where the concentration of surface-active agent at which maximum suspendability was observed held constant (4 mg./ml.) and varying amounts of sodium chloride were added. The resulting suspension isotherms are shown in Fig. 6, where zinc oxide suspended is plotted as a function of total ionic strength of the system. Dotted and broken lines represent the suspension isotherms for zinc oxide–

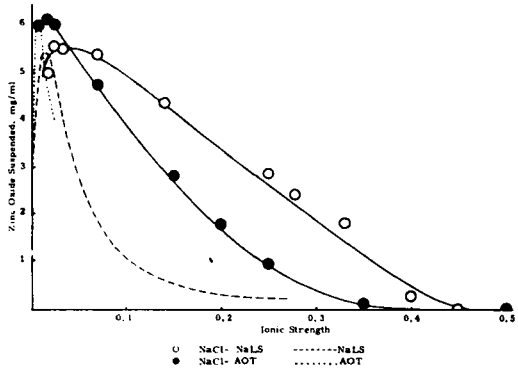

Fig. 6.—Influence of ionic strength on the suspendability of zinc oxide in NaLS and AOT solutions.

AOT and zinc oxide–NaLS, respectively, in the absence of sodium chloride. In order to plot these curves 100% dissociation of the surface-active agent was assumed for the calculation of ionic strength, thus these curves are displaced slightly to the right along the ionic strength axis.

It is seen that although suspendability decreased upon addition of increasing amounts of electrolyte, it did not fall off as rapidly as in the case of addition of excess surface-active agent. The major effect brought about by the presence of electrolyte would be the reduction in zeta potential and, perhaps at higher ionic strength, some dehydration of the interfacial film. It would thus appear that excess anionic surface-active agent in the system facilitates aggregation by some mechanism in addition to reduction of zeta potential. It seems reasonable to expect this mechanism to be film to film interactions enhanced by multilayer adsorption of surface-active agent at concentrations in excess of the critical micelle concentration.

As a result of these studies it may be deduced that both reduction in zeta potential and the presence of excess surface-active agent contribute to the deaggregation and aggregation of suspended particles. The relative significance of each in the total behavior shown can only be determined through direct measurement of aggregation in these systems under controlled environmental conditions.

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Effect of Neostigmine on the Tissue Concentration of Antibiotic in Streptomycin Treated Rats

By VERNON A. GREEN†

Increased streptomycin levels in rat skeletal muscle tissue were observed after the administration of neostigmine methylsulfate alone and in combination with atropine. Acetylcholine and methacholine bromide were administered to determine if the increase in antibiotic concentration was due to vasodilation or to some other action of the anticholinesterase.

THIS AUTHOR (1–3) has reported both a potentiation of activity and an increase in the rate of tissue penetration of some agents which have been administered after cholinesterase inhibitors. Similarly, Greig (4) has noted an increased barbital concentration in mouse brain when the barbiturate was administered shortly after an anticholinesterase agent. The purpose of this investigation was to ascertain the concentration of streptomycin in skeletal muscle and blood serum of rats which received the antibiotic after the administration of either neostigmine methylsulfate alone and in combination with atropine sulfate or the choline esters—acetylcholine and methacholine bromide.

Streptomycin levels in muscle extracts and blood serum were determined by a bacteriological cup method. A standard curve was obtained after the method of Grove and Randall (5), with reference standards from 0.1 to 10 mcg. The curve was then used to determine the streptomycin level in the rat tissue.

EXPERIMENTAL

Procedure for Determining the Streptomycin Level in Rat Tissue (I).—Albino rats used in this study were selected without regard for age, weight, or sex. Control and experimental animals were studied concurrently in each procedure.

The experimental animals were administered 0.0004 mg./Gm. neostigmine subcutaneously. Thirty minutes after the injection of the cholinesterase inhibitor into the experimental animals, 40 mg./Kg. of streptomycin was administered intramuscularly into the right rear thighs of both the control and experimental animals.

Three hours after the administration of the streptomycin, the animals were etherized and sacrificed by severance of both common carotid arteries, whereupon the blood was collected in centrifuge tubes and refrigerated. The skin was then removed from the left rear leg and the muscle tissue removed. After washing the tissue to remove any remaining blood,[1] the fascia and fat were removed and 20 Gm. of the remaining muscle tissue added to 100 ml. of the cold phosphate buffer,[2] and homogenized for 15 minutes at high speed in a Waring Blendor. The homogenate was then diluted to 200 ml. with cold phosphate buffer to be used for testing.

Three cups on each of three agar plates to be used in the test were filled with 0.2 ml. of the homogenate and the other 3 cups filled with 0.2 ml. of a known concentration of streptomycin, 1 mcg./ml. of tissue extract, as a control. The plates were covered and incubated for 18 hours at 37° at which time the diameters[3] of the inhibitory zone for each cup of the homogenate and control were noted. The readings were then corrected to the 1 mcg./ml. reference point by the same procedure used in establishing the standard curve and the corrected zone measurements compared to the standard curve in order to interpret the number of micrograms of streptomycin per milliliter in the experimental test tissue homogenates. Because a dilution factor of the tissue was involved, 20 Gm. in 200 ml., the figures obtained from the standard curve were multiplied by 10 and the milligram concentration of streptomycin per gram of tissue was reported.

The Determination of the Effects of the Cholinesterase Inhibitor on the Serum Levels of Streptomycin in Rats (II).—The blood collected was allowed to clot in the refrigerator; the clot was then loosened with a sterile loop and the tube centrifuged at high speed about 25,000 r.p.m. for 7 minutes. After centrifuging, the serum was pipetted off and diluted from 1 part serum up to 4 parts with phosphate buffer. The streptomycin concentration of the serum was then determined by the same method as the antibiotic concentration of the muscle tissue, using 0.2 ml. of 10 mcg./ml. solution of streptomycin in the control cups as the control. The readings of the zones of inhibition and corrections were made after the same method used in part I, but using the 10 mcg./ml. reference point as the correction standard.

The Determination of the Effects of Atropine on

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[1] The wash liquid was the same phosphate buffer that was used for the dilution of the extract—dibasic potassium phosphate, 16.73 Gm. and monobasic potassium phosphate, 0.523 Gm. per 1000 ml. adjusted to pH 8.

[2] Cold phosphate buffer was used since the liquid in the blender had a tendency to heat up, thereby destroying part of the streptomycin activity.

[3] Since the zones of inhibition were irregular, six different measurements of the diameter for each zone were made and the average recorded as the diameter of the inhibitory zone.

the Streptomycin Levels in Neostigmine-treated and Untreated Rats (III).—Experimental and control animals were prepared and medicated in the same manner as in part I, except that the controls received 8 mg. of atropine sulfate subcutaneously 30 minutes prior to streptomycin and the experimental animals received 8 mg. of atropine sulfate subcutaneously with the neostigmine prior to streptomycin. Three hours after the administration of streptomycin, the animals were sacrificed and the muscle tissue and blood serum used for the determination of the streptomycin level as in parts I and II. The readings of the zones of bacterial inhibition, and corrections, were made as in parts I and II.

The Determination of the Effects of Methacholine and Acetylcholine on the Streptomycin Level in Rat Tissue (IV).—A vasodilator, methacholine bromide 0.05 mg./Kg. intramuscularly, was given to albino rats 15 minutes prior to 40 mg./Kg. of streptomycin. After 3 hours, the animals were sacrificed and the blood serum and muscle tissue collected and tested as in parts I and II above. Streptomycin tissue levels were determined and corrected for reporting as described above.

When acetylcholine was administered with neostigmine at a dose level of 0.05 mg./Kg., all animals succumbed, but by decreasing the acetylcholine dose to 0.01 mg./Kg. with neostigmine prior to streptomycin, the animals survived. The muscle tissue and blood serum in this group were checked as above.

RESULTS AND DISCUSSION

Streptomycin Concentration in Rat Tissue-Controls.—Six rats were given streptomycin 40 mg./Kg. intramuscularly; after 3 hours the animals were sacrificed and the blood and muscle tissue checked for antibiotic activity by the cup method.

The homogenate of the muscle tissue, 1:10 dilution, gave no inhibitory zones, indicating that the concentration of streptomycin was less than 0.005 mg. per gram of tissue by this method. Blood serum levels were checked and found to be, for the average of the six animals, 0.0402 mg./ml. These findings were recorded to be used as the basis of comparison for animals receiving prior medications.

Streptomycin Concentration in Tissue and Blood Serum of Rats Pretreated with Neostigmine.—Neostigmine 0.4 mg./Kg. was given subcutaneously to six rats 30 minutes prior to 40 mg./Kg. of intramuscular streptomycin. Three hours after streptomycin administration the animals were sacrificed; the blood and tissue were collected and tested for antibiotic activity as described above.

The muscle tissue showed streptomycin activity equal to 0.0159 mg./Gm. which was more than the controls, since the controls showed less than 0.005 mg. per gram. The serum level of streptomycin as determined in these tests showed only 0.0245 mg./ml., a 0.0157 mg./ml. decrease from that of the control animals.

Streptomycin Concentration in Rats Premedicated with Atropine and Neostigmine.—Eight milligrams of atropine sulfate plus neostigmine methylsulfate were administered 30 minutes prior to 40 mg./Kg of intramuscular streptomycin to six rats. Three hours after the streptomycin administration, the animals were sacrificed, the blood and muscle tissue collected and tested as above to determine the antibiotic activity.

One group of plates with muscle tissue of one animal showed no inhibition beyond the cups, but in the other five there were clearly defined zones of inhibition which gave an average reading of 0.0123 mg. of streptomycin per gram of muscle tissue. The blood serum level gave an average of 0.0299 mg. per ml for the six animals tested. These findings were similar to those for neostigmine alone.

Atropine 8 mg. per animal is a dose of atropine that should prevent the vasodilating effects of neostigmine, even though the rat is quite tolerant to the belladonna alkaloids. Because the administration of atropine did not change appreciably the tissue levels of streptomycin in the above animals, it appears evident that the increase in tissue levels of the antibiotic in neostigmine pretreated rats is not primarily due to vasodilation.

Streptomycin Concentration in Tissue of Rats Pretreated with Atropine Sulfate.—Six rats of varying ages, weights, and sex were administered subcutaneously 8 mg. of atropine sulfate 30 minutes before streptomycin 40 mg./Kg. intramuscularly. Three hours after the streptomycin injections, the animals were sacrificed and the blood and tissue collected for testing.

Five of the animals showed concentrations of streptomycin too small to read by this method, *i.e.*, less than 0.005 mg. per gram of tissue. One animal, however, showed slight inhibitory zones about the test cups, but the 1 mcg./ml. reference cups were much below the average which increased the corrected reading to a point that it could be estimated by extending the slope of the standard curve. However, the concentration of the antibiotic was still below 0.005 mg./Gm.

The blood serum level of streptomycin in the atropinized animals was 0.0216 mg./ml., which was less than the control but near that for animals pretreated with neostigmine alone or neostigmine and atropine combined.

Streptomycin Concentration in Rats Pretreated with Methacholine and Acetylcholine.—Methacholine bromide 0.05 mg./Kg. was administered to 4 rats subcutaneously and followed immediately by streptomycin 40 mg./Kg. intramuscularly. After 3 hours, the tissue and blood serum were collected from the sacrificed animals, as above, and tested for streptomycin activity.

The muscle tissue in methacholine pretreated rats showed a tissue level of 0.0076 mg./Gm. and the blood serum 0.02727 mg./ml. The muscle tissue antibiotic level was slightly more than the controls, and the blood serum level was much less than the serum level of the controls, but about the same as that of neostigmine treated and atropinized animals in this study.

Acetylcholine bromide 0.05 mg./Kg. was administered to four rats subcutaneously, followed immediately by streptomycin 40 mg./Kg. After 3 hours the animals were sacrificed; the tissue and blood serum collected and tested as above to determine the antibiotic level.

The muscle tissue showed a tissue level of streptomycin of 0.0068 mg./Gm. and the serum 0.0328 mg./ml. which was about the same as metha-

STREPTOMYCIN IN RAT TISSUE[a]

	Concn., Blood Serum, mg./ml.	Concn., Skeletal Muscle, mg./Gm.
Streptomycin alone	0.0402	Less than 0.005
Streptomycin and neostigmine	0.0245	0.0159
Streptomycin, neostigmine, and atropine	0.0299	0.0123
Streptomycin and atropine	0.0216	Less than 0.005
Streptomycin and methacholine	0.0272	0.0076
Streptomycin and acetylcholine	0.0328	0.0068

[a] The above was determined from tissue obtained from animals three hours after administration of 40 mg./Kg. of the antibiotic.

choline for the tissue, but a higher level for the serum.

This study of the effects of methacholine and acetylcholine on the tissue levels of streptomycin indicates that vasodilation produced by these agents did not cause as appreciable a rise in the tissue level of the antibiotic as does neostigmine alone, or in combination with atropine. If vasodilation were the only factor involved in the increase in the tissue level of streptomycin brought about by the neostigmine, methacholine and acetylcholine should have given an increase in the tissue level of the antibiotic equal to that produced by neostigmine.

SUMMARY AND CONCLUSION

The cup method using agar plates seeded with *B. subtilis* was used to determine the streptomycin concentration of the tissue and blood serum of rats receiving 40 mg./Kg. of streptomycin. Additional tissue and blood levels of streptomycin were determined on rats premedicated with neostigmine, neostigmine and atropine combined, atropine, methacholine, or acetylcholine.

Animals premedicated with neostigmine and neostigmine plus atropine, showed tissue levels of 0.0159 mg./Gm. and 0.0123 mg./Gm., respectively, which represented increases over the controls (streptomycin level less than 0.005 mg./Gm.). Also with the increased tissue level there was a decrease in the blood serum level.

Attempts to increase the rat tissue levels of antibiotic by the use of the vasodilators, methacholine and acetylcholine 0.05 mg./Kg., resulted in tissue levels of 0.0076 mg./Gm. and 0.0068 mg./Gm., respectively.[4] These tissue levels were slightly greater than the controls, but much less than the levels in neostigmine, or neostigmine and atropine combined, pretreated animals.

Atropine, 8 mg. per animal, failed to block the tissue level increases of streptomycin produced by neostigmine, although this dose of atropine should have blocked any vasodilating activity of neostigmine.

The observed increases in the rat muscle tissue level of streptomycin must be some action other than that of producing vasodilation by the action of the endogenous acetylcholine that accumulates with neostigmine administration.

From this investigation and previous studies by other authors,[5] it appears that some of the inhibitors of cholinesterase (6) increase the absorption and penetration of certain agents into tissue. Because neostigmine affected increases in the tissue levels of streptomycin, it would appear that it increases the permeability of the skeletal muscle to the antibiotic.

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[4] The above concentrations of streptomycin in methacholine and acetylcholine treated rats is for only half of the test animals since 50% showed tissue concentration less than 0.005 mg./Gm.

[5] Increases in streptomycin penetration in the CSF of dogs reported to the Scientific Section, A.PH.A., Washington, D. C. meeting, 1960.

Quantitative Spectrophotometric Papergram Assays I

Tubercidin

By T. F. BRODASKY and W. L. LUMMIS

The paper chromatographic characteristics of the cytotoxic agent tubercidin in a number of solvents is reported. The technique for the production of bioautographs on Eagle's KB carcinoma cells is described. A study of the quantitation of tubercidin on KB cells and on papergrams by ultraviolet analysis was undertaken. Using the same chromatograms, ultraviolet analysis followed by bioautographic assay allowed the compilation of data which were statistically analyzed by the pairing technique. This indicated that the two methods were equivalent; however, the ultraviolet assay is the method of choice inasmuch as it requires only half the time required for the biological method.

BECAUSE of the inherent nature of pharmaceutical compounds, laboratories rely upon biological methods to detect, identify, and assay new drug candidates. This often requires handling pathogenic materials or systems requiring lengthy procedures for quantitative evaluation. It would be advantageous, once biological activity has been established, to use a more direct method, one not based on a biological process but which compared favorably with the biological procedure in regard to quantitative features. In this regard, a series of papers (1, 2) has been written concerning the statistical comparison of biological and spectrophotometric assay methods. The principal aim of the work is to establish the usefulness of existing nonbiological techniques in quantitating biological materials and to point out, in a limited way, the nature of these techniques with regard to accuracy, precision, and time comsumption. Three compounds, each presented in a separate paper, have been chosen because of their dissimilar biological nature and response to light absorption techniques.

Considerable work has been done on quantitating papergrams by ultraviolet spectroscopy (3) and a number of unique devices for scanning papergrams has been developed (4). This paper describes the use of spectrophotometry to follow a biologically active compound. Since paper chromatography is a basic tool in the study of pharmaceuticals, the quantitative absorption of ultraviolet radiation by tubercidin (5) on chromatographic paper was studied. Tubercidin was chosen because it represents a class of compounds principally active on carcinoma tissue culture. The use of this system entails some difficulty, and the ability to detect tubercidin by a physical method would to some extent alleviate this problem. The technique of producing bioautograms of this antibiotic with human carcinoma cells in tissue culture and subsequent development of a quantitative papergram assay are also described. An ultraviolet quantitative papergram assay was developed using the 272 mμ absorption peak of tubercidin.

A statistical comparison of the two assays was made to determine if the absorption and scattering errors inherent in the spectrophotometric papergram assay would limit the usefulness of this method.

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EXPERIMENTAL

Bioautograms and Quantitative Papergram Assay.—Whatman No. 1 sheets, 15 × 44 cm., were spotted with 100-μl. samples of fermentation liquor containing tubercidin. The papergrams were developed 16 hours (5 hours, for V and VI) descending in the six solvent systems listed in Table I in a sealed chromatographic chamber. After development, the strips were air-dried (System III strips were neutralized in an ammonia atmosphere for 10 min.), then plated on 19 × 50 cm. trays containing 125 ml. of modified Miyamura agar seeded with 0.2 mg. Eagle's KB epidermoid carcinoma cell protein per ml. (6). The trays were incubated at 37° for 16 hours with the strips in contact with the agar. The zones of activities on the trays were detected by spraying the agar with an 0.4% solution of 2,5-dichlorophenol-

TABLE I.—PAPERGRAM SOLVENT SYSTEMS

System	Composition
I	1-butanol:water::84:16 (v/v)
II	1-butanol:water::84:16 (v/v) plus 0.25% *p*-toluenesulfonic acid (w/v)
III	1-butanol:acetic acid:water::2:1:1 (v/v/v)
IV	1-butanol:water::84:16 (v/v) plus 0.2% (v) piperidine
V	1-butanol:water::4:96 (v/v)
VI	1-butanol:water::4:96 plus 0.25% *p*-toluenesulfonic acid (w/v)

indophenol in methanol-saline (1:20 by volume). Within 1 hour, during which time the unaffected cells reduced the blue dye, the zones of activity appeared dark blue against a colorless or light blue background. None of the solvents resulted in inhibition of KB cells as shown by plating strips washed in each of the mobile phases.

The KB cell assay was developed using essentially the procedure described above. The crystalline standard (purity >95%) was spotted at 10, 20, 40, and 60 mcg. per 0.5 in. strip of Whatman No. 1 paper and developed in solvent system IV. A set of standard strips were developed for each assay tray. The maximum width of the zones of activity were plotted against the log of the dose per strip to construct a standard assay curve.

Ultraviolet Quantitative Papergram Assay.— Using measured volumes of a 10 mcg./ml. dimethylformamide solution, a 0.5 in. strip of Whatman No. 1 paper was spotted with 50 mcg. of tubercidin (purity >95%) and developed descending in system IV for 16 hours. After drying, the strip was placed on an ultraviolet scanning box and the center of the tubercidin zone marked. This zone was then scanned on a Cary spectrophotometer from 365 to 210 mμ with a sheet of Whatman No. 1 paper in the reference beam. Two maxima were noted, 238 and 272 mμ.

Strips spotted with 10, 20, 40, and 60 mcg. per strip of tubercidin were developed descending in system IV for 16 hours. After scanning the strips at 272 mμ using a strip scanner, the areas under the response peaks were calculated by the height times the half band width method and plotted against log dose per strip to construct a standard curve.

RESULTS AND DISCUSSION

The chromatographic pattern for tubercidin in six solvent systems on Eagle's KB cells is shown in Fig. 1. This pattern correlates with the patterns on *Bacillus subtilis* and *Proteus vulgaris*. The activity on the latter organisms was considerably weaker than on KB tissue culture and for this reason the bioassay was developed on KB cells. The 272 mμ peak of tubercidin was chosen for the ultraviolet papergram assay for several reasons. The 272 mμ peak was more intense than the 238 mμ peak, $a_{272} = 33$, $a_{238} = 8$. The 238 mμ peak failed to give a linear relationship between log dose per strip and either optical density or area of response peaks.

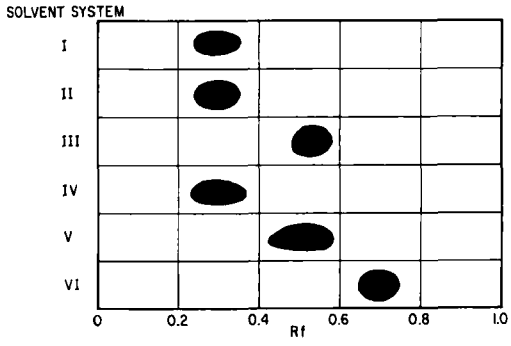

Fig. 1.—KB tissue culture papergram pattern of crystalline tubercidin.

The 238 mμ peak also showed an intensity variation depending upon the pH of the solvent system used in development. The 272 mμ peak did not display any of these variations.

The standard assay curves for the biological and ultraviolet method appear in Figs. 2 and 3, respectively. The points represent the average of 7 determinations and the indices of precision (S/b) of the curves for the biological and ultraviolet method are 0.22 and 0.06. The 95% confidence limits about the points are indicated. Data have been obtained for a limited number of runs indicating the accuracy of the assay methods. Tubercidin was chromatographed in system IV at known doses in the presence of compounds which are coproduced with this antibiotic. Strips were first analyzed by ultraviolet spectroscopy and subsequently assayed by the tissue culture process. The errors were -6.4 ± 5.3 and $25 \pm 34\%$, respectively. The large errors incurred in the tissue culture procedure can be traced to variable sensitivity of the assay organism

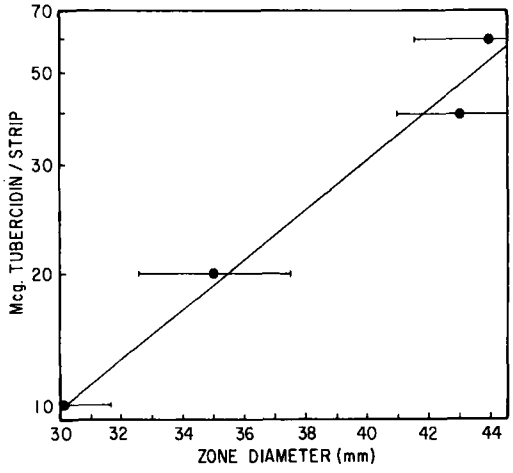

Fig. 2.—Tissue culture assay standard curve for tubercidin.

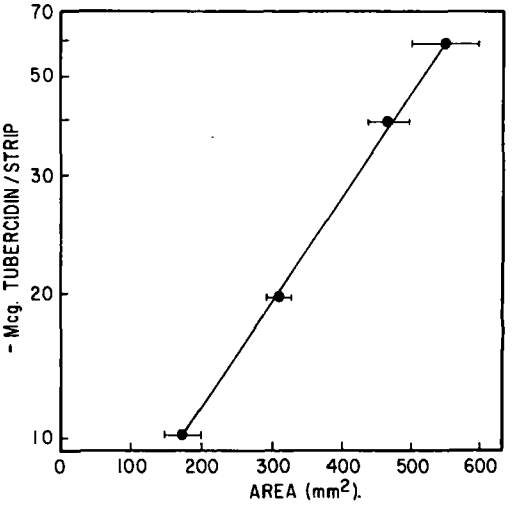

Fig. 3.—Ultraviolet assay standard curve for tubercidin at 272 mμ.

in this early stage of development. A great deal of care must be taken in preparing the trays since such factors as agar thickness can result in a large day-to-day variation in response.

The precision of the assay procedures is given in Table II. This data was accumulated from assays of

TABLE II.—PRECISION OF ASSAY METHODS

	Mcg./mg. of Tubercidin		
	Ultraviolet	d[a]	Biological
	930	−100	1030
	1050	− 50	1100
	960	160	800
	900	120	1020
	1050	30	1020
	1000	− 20	1020
	900	50	850
	1200	180	1020
Mean	999	16	983
S	101	109	102
95% Confidence limits	±85 mcg./mg.		±86 mcg./mg.

[a] d = differences between paired assay values.

crystalline tubercidin (purity >95%) performed first by ultraviolet analysis and then, using the same strips, by the tissue culture method. This was done to allow testing of the equality of the means of the population samples by the pairing technique (7). The difference between paired values of each set of assay results represents a random sample of a population of unknown variance. The population is assumed to have a normal distribution. The pairing procedure allows testing of the mean of the differences for zero and therefore whether the procedures are equivalent. The mean is tested using the t distribution statistic, $t = [\bar{d} - (\mu_1 - \mu_2)]/(Sd/\sqrt{N})$. Assuming that $\mu_1 = \mu_2$, the value of t equals 0.41 and one rejects the hypothesis that $\mu_1 = \mu_2$ only if t is greater than +2.36 or less than −2.36 at a 5% level of significance. The 95% confidence limits shown in Table II were obtained using the statistics for a t distribution $(\bar{x} + (t_{\alpha/2}S)/\sqrt{N}; \bar{x} + (t_{1-\alpha/2}S/\sqrt{N})$ (8). The data indicate a favorable agreement between the two methods. Since the incubation of biological trays is eliminated in the spectrophotometric method, the time necessary for assays is reduced by one-half.

The use of Whatman No. 1 paper in the reference beam of the spectrophotometer during the recording of the response curves does not eliminate the errors due to scattering and absorption by the paper but the inherent advantages of the method offset in part the lack of ultimate quantitation. The spectrophotometric method has been successfully used to estimate the amount of tubercidin in fermentation cultures down to 38 mcg./ml. of culture. In the assay of crystalline preparations the ultraviolet method is capable of detecting biologically inactive materials. This eliminates the inherent error in the biological assay of basing assays on materials which appear pure by biological standards but may actually contain biologically inactive material. For this reason, preparative samples can be selected by the ultraviolet method for standards which are of the highest purity.

SUMMARY

Tubercidin has been chromatographed in a number of solvents to obtain a pattern of Eagle's carcinoma cells. The chromatographic pattern was found to correlate with the *B. subtilis* and *P. vulgaris* patterns although the latter were considerably weaker.

Quantitative papergram assays were developed on carcinoma tissue culture and by using spectrophotometric techniques on papergrams. A statistical analysis of both methods indicates that the assays are essentially equal. However, the spectrophotometric method is more accurate and reduces the assay time by one half.

GLOSSARY OF TERMS

μ_1—Mean of the population values for the ultraviolet assay.
μ_2—Mean of the population values for the biological assay.
$\bar{d}$—Mean of the differences between the paired values (assays).
S_d—Standard deviation of the differences between the paired values (assays).
α—Probability of rejecting true hypotheses; level of significance.
N—The number of samples (assays).
S—Standard deviation of the samples (assays).
$\bar{x}$—The mean of the samples (assay).
$t_{\alpha/2}$; $t_{1-\alpha/2}$—Limits of the critical region for a t distribution as determined by α.
b—Slope of standard curves.

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Quantitative Spectrophotometric Papergram Assays II

Sparsomycin, A New Antitumor Antibiotic

By T. F. BRODASKY

The paper chromatographic characteristics of a new cytotoxic agent, sparsomycin, on a variety of solvent systems are reported. The chromatographic pattern of this antibiotic on Eagle's KB human epidermoid carcinoma cells in agar was found to correlate with the pattern on *Bacillus subtilis* UC-564. A quantitative papergram assay was developed on agar seeded with *B. subtilis* and compared statistically with a quantitative papergram assay based on the ultraviolet absorption of sparsomycin. The equivalence of the assay methods was determined by testing the equality of the means of the population of assay values using the pairing technique. The effect of the edges of the papergram on the chromatographic zone and its influence on the ultraviolet assay curve are described. There is a favorable agreement between the assays; however, the ultraviolet method reduces the assay time by 50% in comparison to the biological method.

A NEW cytotoxic agent, sparsomycin,[1] has been isolated from *Streptomyces sparsogenes* culture. This paper describes the paper chromatographic characteristics of sparsomycin and the development of a biological and physical quantitative papergram assay. Fermentation, isolation, and characterization studies are described in other papers (1, 2).

Since sparsomycin is coproduced with three other biologically active components, a disk plate assay is not feasible except as a measure of total bioactivity. Paper chromatography in suitable solvent systems yields resolved zones and allows quantitative evaluation of doses of individual components on the papergram. A statistical comparison between the two assays was made to determine the most rapid and reliable means of estimating yields in culture media and purity of preparative samples.

EXPERIMENTAL

Whatman No. 1 paper strips, 15 × 44 cm., containing 10 mcg. of sparsomycin per strip were developed descending for 16 hours (5 hours, in the case of V and VI), using the solvent systems listed in Table I. After a one-hour drying period, the strips were plated on 19 × 50 cm. trays containing 0.2 mg. of Eagle's KB cell protein per ml. of modified Miyamura agar (3). The trays were incubated for 16 hours at 37° with the strips remaining in contact with the agar. Upon removal of the strips, the agar was sprayed with a 0.4% solution of 2,6-dichlorophenol-indophenol in methanol-saline (1:20 by volume). One hour was allowed for dye reduction, during which zones of activity appeared blue against a colorless or very light blue background.

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[1] Sparsomycin was formerly called sparsogenin.

The biological assay was established on agar seeded with *Bacillus subtilis* UC-564. The agar was inoculated with 0.3 ml. per liter of a suspension containing 1.3 × 10^{10} spores/ml. The $^1/_2$-inch Whatman No. 1 paper strips containing 60, 40, 20, and 10 mcg. of sparsomycin per strip (used in both assays) were developed descending for 16 hours in solvent system IV. This system was found to yield the greatest resolution of the various components in the sparsomycin fermentation broths. After one-hour drying, the strips were plated on *B. subtilis* seeded trays and incubated for 16 hours at 30°. Three strips containing assay samples and two strips containing standard (purity > 95%) were plated on each tray. On removal of the strips,

TABLE I.—PAPERGRAM SOLVENT SYSTEMS

System	Composition
I	1-butanol:water::84:16 (v/v)
II	1-butanol:water::84:16 (v/v) plus 0.25 % *p*-toluenesulfonic acid (w/v)
III	1-butanol:acetic acid:water::2:1:1 (v/v/v)
IV	1-butanol:water::84:16 (v/v) plus 0.2% (v) piperidine
V	1-butanol:water::4:96 (v/v)
VI	1-butanol:water::4:96 plus 0.25% *p*-toluenesulfonic acid (w/v)

the diameter of the zones in the R_f 0.1 region were measured. These values were plotted against the log dose per strip to obtain a standard assay curve.

An ultraviolet papergram assay was established on $^1/_2$-inch Whatman No. 1 paper strips using a strip scanner (4) in conjunction with a Cary spectrophotometer. The strips were developed in system IV as before. After drying, the strips were scanned from the origin at 302 mμ with a blank of Whatman No. 1 paper in the reference beam of the spectrophotometer. A set of strips containing standard was run for each group of three unknown samples. Only one peak in the pertinent R_f region appeared and the area under this peak was calculated by the peak height times the half-band width method. The areas were plotted against the log dose per strip to obtain a standard assay curve.

RESULTS AND DISCUSSION

Figure 1 shows the typical six solvent system pattern of sparsomycin against Eagle's KB epidermoid carcinoma cells in agar. A similar pattern was obtained against *B. subtilis* and *Proteus vulgaris*.

Standard curves for the biological and ultraviolet assays appear in Figs. 2 and 3. The points represent an average of 11 determinations and the indices of precision (S/b) of the curves for the biological and ultraviolet methods are 0.047 and 0.072, respectively. The 95% confidence limits for each point are indicated. The 10 mcg. point was not included in the biological curve because of the poor sensitivity of *B. subtilis* to sparsomycin at this level. There was considerably more variation in the higher dose responses in the ultraviolet assay curve than in the biological assay curve and this can be accounted for by irregularities in the chromatographic zone. At high doses on 1/2-inch strips, there is a tendency for some materials to give broad zones exhibiting an effect of the edge of the paper strip. This effect is manifested as a variation in the density of the material across the width of the strip. When the strips are plated on agar, diffusion tends to mask the true density gradient in the zone. When a spectrophotometric determination is made, only the central portion of the strip is scanned because of geometric considerations. Under these conditions, the variation in density of a zone across the width of a strip is manifested as a variation in the area of the response peak. Figure 4 shows an ultraviolet curve indicating the density gradient across a 1/2-inch zone of sparsomycin perpendicular to the direction of development.

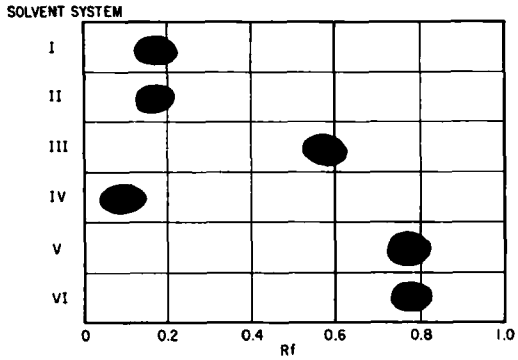

Fig. 1.—Six solvent system patterns of sparsomycin on KB cells.

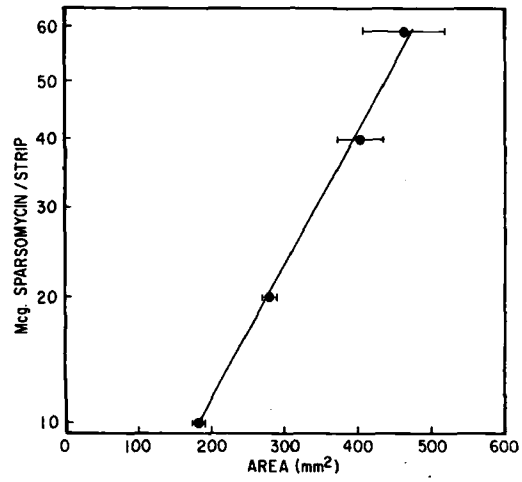

Fig. 2.—Standard *B. subtilis* assay curve for sparsomycin.

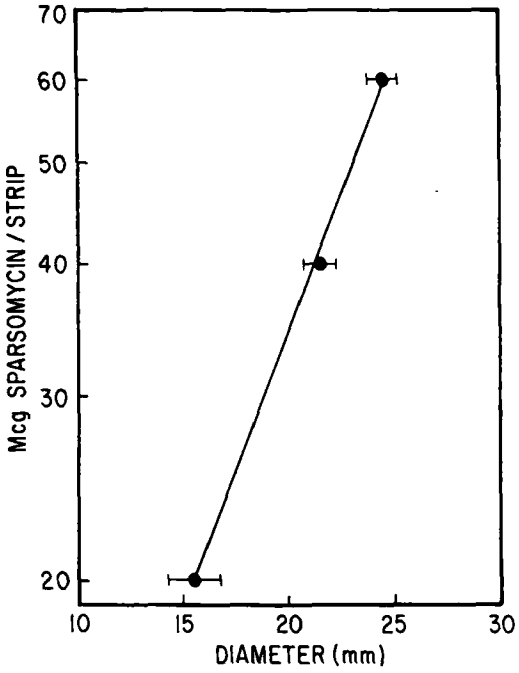

Fig. 3.—Standard ultraviolet assay curve for sparsomycin at 302 mμ.

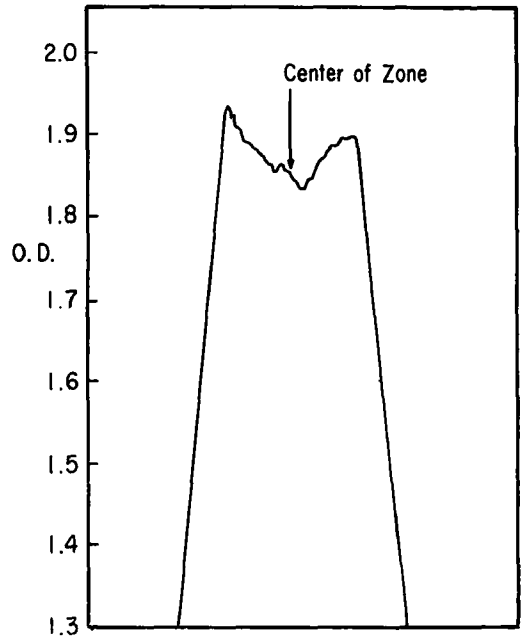

Fig. 4.—Density gradient of sparsomycin zone perpendicular to solvent flow.

Table II summarizes the variation between assay methods. The fact that the mean per cent variation is positive (bioassay with respect to ultraviolet assay) indicates that the ultraviolet assay did not include extraneous material during the recording of the sparsomycin peaks. Large variations in some assay results cannot be explained at this time.

TABLE II.—VARIATION IN UV AND BIOASSAY OF SPARSOMYCIN

Mcg. Sparsomycin/mg. Sample		Variation, %
a UV	*b* Bio	100 (*b* − *a*)/*a*
743	875	+17.8
670	974	+45.3
425	676	+59.7
423	392	− 7.3
676	957	+41.6
1025	1147	+11.6
453	493	+ 8.8
1115	973	−12.7
843	892	+ 5.8
867	910	+ 5.0
785	749	− 4.6
940	934	− 0.6
	Mean	+14.2
	S. D.	22.9

Table III was prepared from data recorded during the evaluation of a crystalline preparation of sparsomycin. The assay results were obtained over a 3-day period using a set of standards for each group of three samples. The 95% confidence limits, determined using a *t* distribution [$\bar{x} + (t_{\alpha/2}\ S)/\sqrt{N}$; $\bar{x} + (t_{1-\alpha/2}\ S)/\sqrt{N}$] (5), indicate considerably greater precision for the ultraviolet assay procedure. The values obtained from ultraviolet assays were consistent with values expected on the basis of ultraviolet and melting point data for sparsomycin. The accuracy of the UV assay was not determined directly. In a previous paper (6), the accuracy of the UV assay for one of the components of a sparsomycin culture in the presence of sparsomycin was found to be −6.3 ± 5.3% at 95% confidence. It is assumed that the sparsomycin assay will be as accurate. Testing for the

TABLE III.—PRECISION OF ASSAY METHODS

Mcg. Sparsomycin/mg. Sample		
UV	*da*	*B. subtilis*
863	− 70	933
950	− 38	988
865	− 60	925
938	+ 38	900
950	+100	850
925	− 42	967
933	− 55	988
900	+ 25	875
890	+ 90	800
900	+ 67	833
Mean 911	5.5	906
S. D. 33	21	66
95% Confidence Limits ± 27 γ/mg.		± 53 γ/mg.

[a] *d* = Difference of paired values of assays.

equality of the mean of the population of values for both assays by the pairing technique indicated that the procedures were equivalent. This is done by determining the value of $t = [\bar{d} - (\mu_1 - \mu_2)]/(S_d/\sqrt{N})$ (7) assuming $\mu_1 - \mu_2$, and comparing it with values obtained from a table giving percentiles for a *t* distribution. The value of *t* was 0.73 and one rejects the hypothesis that μ_1 is equal to μ_2 only if *t* is greater than 2.26 or less than −2.26 at a 5% level of significance.

The data lead to the conclusion that the UV assay compares favorably with the biological method. The UV assay is more rapid than the biological method and eliminates the need for handling biological systems routinely, although it is important to periodically check the ultraviolet method biologically to ascertain that the original bioactivity is being followed.

SUMMARY

Paper chromatographic characteristics of a new cytotoxic agent, sparsomycin, against KB epidermoid carcinoma cells in agar and *B. subtilis* UC-564 are reported.

An ultraviolet assay has been developed and compared statistically with a biological assay. The methods are statistically equivalent with respect to the mean values, but the ultraviolet method is preferred because of greater precision and a reduction in assay time.

GLOSSARY OF TERMS

μ_1–Mean of the population values for the ultraviolet assay.

μ_2–Mean of the population values for the biological assay.

$\bar{d}$–Mean of the differences between the paired values (assays).

S_d–Standard deviation of the differences between the paired values (assays).

α–Probability of rejecting true hypotheses; level of significance.

N–The number of samples (assays).

$t_{\alpha/2}$ − $t_{1-\alpha/2}$–The limits for the critical region, of a *t* distribution, as determined by α.

b–Slope of the standard curve.

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Dissolution Rate Studies II

Dissolution of Particles Under Conditions of Rapid Agitation.

By P. J. NIEBERGALL,† G. MILOSOVICH, and J. E. GOYAN

Dissolution data for sized fractions of three different compounds were obtained using the method of Niebergall and Goyan. The Hixon-Crowell "cube root law" did not describe the data. Deviation from this law was accounted for by deriving a rate expression assuming that the thickness of the diffusion layer was proportional to the square root of the mean volume diameter. Experiments were performed to verify the applicability of the equation so derived.

NOYES AND WHITNEY (1) were the first to report a differential equation relating the rate of dissolution of solid solute to concentration of dissolved solute in the surrounding solvent

$$\frac{dC}{dt} = K(C_s - C) \qquad \text{(Eq. 1)}$$

The rate constant, K, contains several variables which have been the subject of study by succeeding workers. While Noyes and Whitney assumed that the rate would be proportional to surface, it was Brunner and St. Tolloczko (2–5) who showed this dependence and also that K depended upon temperature and rate of stirring. Nernst (6) considered dissolution as a special case of heterogeneous reaction and suggested that Eq. 1 could be written

$$\frac{dC}{dt} = \frac{DA}{V\delta}(C_s - C) \qquad \text{(Eq. 2)}$$

where: D = diffusion coefficient; A = area of the interface; V = volume of solvent; and δ = thickness of a stagnant film of solvent on the solid surface. Brunner (7) showed this rate dependence on the diffusion constant and presented data to show that K was also proportional to (stirring rate)$^{2/3}$.

The Nernst-Brunner layer has been the subject of controversy over the years but, as pointed out by King (8), it is not necessary to the theory that the layer be stagnant on the solid surface. Perhaps the Nernst-Brunner layer may be identical with a hydrodynamic boundary which, rather than being stagnant, merely reflects a velocity gradient between the bulk fluid and the surface of the solid.

In many dissolution studies conditions are chosen such that $DA/V\delta$ remains essentially constant during the experiment. This is accomplished by using single large particles such as compressed tablets or crystal masses grown from the melt. The fraction of the particle which dissolves is so small that surface can be assumed constant as long as the particle remains intact. The thickness of the Nernst-Brunner layer is kept constant by maintaining the position of the particle with respect to the stirrer. When dissolution from powders is studied these conditions may not be obtained. Thus, both surface area and rate of shear on the particles may be expected to change during an experiment.

Hixon and Crowell (9) considered the change in surface as small particles dissolve. They pointed out that the surface area of a particle was proportional to its weight to the $^2/_3$ power, the proportionality constant being composed of density and volume shape factor. If the shape factor remains constant as the particle dissolves the substitution of $W^{2/3}$ for A in Eq. 2 can be made. Then, under the condition that $C_s >> C$ and D, V and δ are constant, the equation can be integrated to give

$$W_o^{1/3} - W_t^{1/3} = kt \qquad \text{(Eq. 3)}$$

where: W_o is the initial weight of solute, W_t is the weight at time t. Equation 3 may also be written

$$W_o^{1/3} - W_t^{1/3} = \frac{k'N^{1/3}DC_st}{\delta} \qquad \text{(Eq. 4)}$$

for N particles, where k' is a constant containing surface and volume shape factors and particle density. The validity of Eqs. 3 and 4 depend on the constancy of k and k'. Shape factors for cubic or spherical particles will remain constant as long as the particles dissolve equally from all sides. This is not true for other particle shapes, and the equations would not be expected to hold for nonequant habits after dissolution has occurred.

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While δ was ignored or assumed constant by Hixon and Crowell, inspection of their data (10) shows that their rate constants increased as dissolution proceeded. Since they were working with cubic salt crystals, this drift probably was not due to shape-factor effects. It could be due, however, to a δ dependence on particle size.

Wilhelm, *et al.* (11), reported an empirical relationship between δ and particle size

$$\frac{D}{\delta} = f(d^{1/4})$$

where the symbols have their usual meaning. Their results, however, predict that the Hixon-Crowell rate constant would decrease with dissolution.

This effect has also been reported in studies on crystal growth (12) under diffusion control. McCabe and Stevens (13) verified that the cube root law applies for growth of various sized crystals under conditions of constant shear. On this basis they postulated that larger crystals grow faster because of a thinner Nernst-Brunner layer due to greater shear imposed on the larger particles in a slowly stirred system. This leads to the analogous situation of dissolution, *i.e.*, larger particles dissolving faster because of thinner diffusion layer.

Thus, a review of the literature indicates that the dissolution process for small particles is still not theoretically well defined. It is most apparent that the influence of agitation is important, yet the effect of various degrees of agitation is still unanswered. Certainly, if larger particles dissolve faster under conditions of agitation present in the intestinal tract, one must question the practice of reducing size to effect faster dissolution of drug particles for oral dosage.

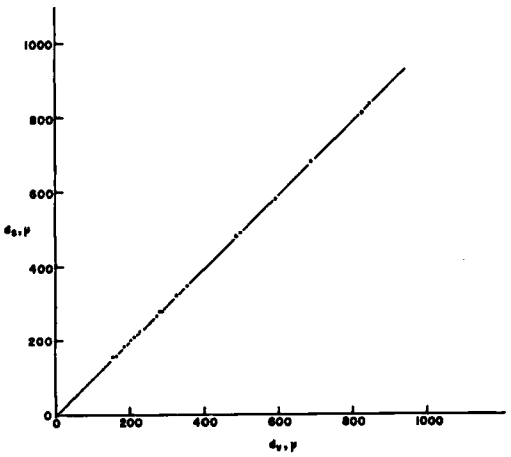

Fig. 1.—Plot of mean surface diameter *vs.* mean volume diameter for all sized fractions used in this study. Slope = 0.99.

This paper reports the initial investigation on the effect of stirring on the dissolution process for small particles. It was the objective of this study to test the applicability of the cube root law to dissolution of particles moving freely in rapidly stirred systems.

EXPERIMENTAL[1]

The continuous recording spectrophotometric procedure described by Niebergall and Goyan (14) was used in this study with one modification. The pump introduced a considerable amount of heat into the solution and made temperature control in the dissolution vessel inadequate. It was decided, therefore, to collect the returning solution in a graduated cylinder. It was then necessary to make corrections for volume changes and loss of solute to the cylinder. These corrections were simplified by the fact that the concentration in the dissolution

[1] Equation 5 is more correctly written

$$W_o - W_t = C_t [V_o - ft] + \int_o^t \frac{dC_t}{dt} ft\, dt \quad \text{(Eq. 6)}$$

which is equivalent to

$$CV_o = C_t [V_o - ft] + f \int_o^t \frac{dC_t}{dt} t\, dt \quad \text{(Eq. 7)}$$

Differentiating Eq. 7 with respect to time

$$V_o \frac{dC}{dt} = [V_o - ft] \frac{dC_t}{dt} - f C_t + f t \frac{dC_t}{dt}$$

$$= V_o \frac{dC_t}{dt} - C_t$$

and

$$\frac{dC_t}{dt} = \frac{dC}{dt} + \frac{fC_t}{V_o} \quad \text{(Eq. 8)}$$

Now, squaring Eq. 14 and rearranging gives

$$W_o - W_t = 2 W_o^{1/2} k'' N^{1/2} t - (k'')^2 N t^2$$

or

$$C V_o = 2 W_o^{1/2} k'' N^{1/2} t - (k'')^2 N t^2$$

Thus,

$$\frac{dC}{dt} = \frac{2 W_o^{1/2} k'' N^{1/2}}{V_o} - \frac{2(k'')^2 N t}{V_o} \quad \text{(Eq. 9)}$$

Combining Eqs. 8 and 9 gives

$$\frac{dC_t}{dt} = \frac{2 W_o^{1/2} k'' N^{1/2}}{V_o} - \frac{2(k'')^2 N t}{V_o} + \frac{fC_t}{V_o} \quad \text{(Eq. 10)}$$

Under the experimental condition that

$$2 (k'')^2 N t = f C_t$$

dC_t/dt would be constant and a plot of C_t *versus* time would be linear. If f is approximately constant as was the case in these experiments, a linear plot would be expected only in the case where

$$\left(\text{Since } C_t = \int_0^t \frac{2 W_o^{1/2} k'' N^{1/2}}{V_o}\right)$$

However, at the relatively small t and C_t values and the average flow rate used in this study, the deviation from linearity is negligible. Nonlinear plots were evident only with the smallest size materials and in these cases the corrections were made using Eq. 7 and graphical integration.

Note that data giving a linear C_t *vs.* t plot may not necessarily be described by Eq. 14, nor will data fitting Eq. 14 necessarily give linear C_t *vs.* t plots. The results reported in this paper were obtained under a fortuitous choice of experimental conditions which gave good correlation between Eqs. 5 and 14.

vessel increased linearly with time. The amount of solute dissolved in time, t, is then given as

$$W_o - W_t = C_t(V_o - ft) + {}^1/_2kft^2 \quad \text{(Eq. 5)}$$

where: C_t = concentration in the vessel at time, t; V_o = initial volume in the vessel; f = flow rate determined by timing the flow into the cylinder; and $k = dC_t/dt$, obtained by plotting concentration against time.

After this modification was made, continuous temperature recording using a thermistor in a wheatstone bridge circuit and a Varian strip-chart recorder showed a maximum temperature variation of $-0.1°$.

The three materials used in this study were selected on the basis of solid state, particle shape, and suitable spectral curves. Potassium dichromate (absorption minimum 317.5 mμ) reagent grade was obtained as equant triclinic prisms. Salicylamide U.S.P. (absorption minimum 262.5 mμ) was spray-congealed to obtain spherical aggregates of microcrystals. Potassium ferricyanide (absorption minimum 418 mμ) reagent grade was obtained as equant rectangular prisms (maximum length to width ratio of about 1.5) with rounded edges. While the latter particles were not as symmetrical as the others it was felt that for the size ranges used the amount of dissolution required for a determination would not significantly alter the shape factor. These materials were sieved three times to obtain sized fractions in which particle sizes were then determined microscopically. Figure 1 is a plot of d_s against d_v, and shows by its slope (0.99) that these fractions were essentially monodisperse (monodisperse slope = 1.00). Size data is presented in Table I.

The solvent selected for these three materials was 50% v/v aqueous ethanol, 750 ml. being used in each run. This solvent was chosen because it wetted the particles and was not a "good" solvent for these compounds (solubility ~2–4%). One-gram samples of each size fraction were used and the stirrer was operated at 500 r.p.m. This speed was chosen as being rapid enough to insure complete suspension of the particles, instantaneous mixing, and representative sampling, yet slow enough that the dissolution process was diffusion controlled. This is seen in the plot of rate constant against r.p.m. in Fig. 2.

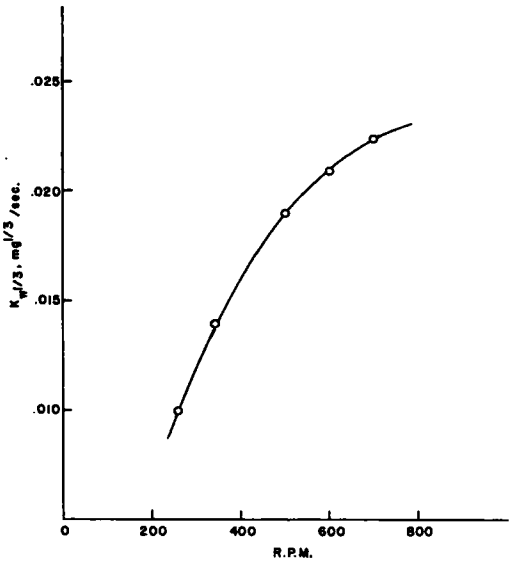

Fig. 2.—Effect of stirrer r.p.m. on the Hixon-Crowell rate constant for dissolution of 681μ potassium dichromate. Complete suspension of particles at 400 r.p.m. T = 24.8°.

The data were plotted initially as $\Delta W^{1/3}$ *vs.* t and good straight lines were obtained in all cases. The slopes of these lines would then be equal to $(k'N^{1/3}\cdot DC_s)/\delta$ as seen from Eq. 4 and are given as $k_W^{1/3}$ in Table I. Since linear plots were obtained, even though particle size changed with time, it would appear that δ does not depend on size. To test this further, the rate constants for different sized fractions were plotted against the reciprocal of the respective mean volume diameters. The expected linear relationship was not obtained. A logarithmic plot of rate constant *vs.* d_v showed for all the materials that the rate constant depended on $d_v^{-3/2}$, Fig. 3.

One way that this relationship may be obtained from Eq. 4 is to set δ proportional to $d_v^{1/2}$. Equation 4 can then be modified as follows:

Table I.—Size Data and Dissolution Rate Constants for the Three Materials Used in this Study

Material	Screen Size	d_s, μ	d_v	$K_W^{1/3}$, mg.$^{1/3}$/sec.	$K_W^{1/2}$, mg.$^{1/2}$/sec.
Spray-congealed salicylamide, 24.8°	50–60	278	281	0.108	0.482
	60–70	227	230	0.157	0.691
	70–80	208	211	0.172	0.750
	80–100	184	187	0.206	0.880
	100–140	158	159	0.245	1.02
Potassium dichromate, 23.4°	20–30	838	846	0.0063	0.030
	30–40	681	686	0.0088	0.042
	40–50	482	489	0.0122	0.056
	50–70	322	326	0.0246	0.113
	70–100	269	272	0.0363	0.163
	100–140	209	213	0.0503	0.230
	140–200	159	164	0.0713	0.330
Potassium ferricyanide, 23.4°	20–30	814	829	0.023	0.110
	30–40	581	592	0.035	0.187
	40–50	490	500	0.049	0.220
	50–70	349	355	0.080	0.347
	70–100	280	288	0.112	0.503
	100–140	201	207	0.209	0.890

$$-\frac{dW}{dt} = \frac{3k'N^{1/3}DC_sW^{2/3}}{\delta} \quad \text{(Eq. 11)}$$

since $d_v^3 = \frac{W}{\alpha_v N}$, α_v being the volume shape factor

$$\delta = k\,d_v^{1/3} = \frac{k\,W^{1/6}}{\alpha_v^{1/6}N^{1/6}} \quad \text{(Eq. 12)}$$

combining Eqs. 11 and 12

$$-\frac{dW}{dt} = \frac{3k'\alpha_v^{1/6}N^{1/2}DC_sW^{1/2}}{k} \quad \text{(Eq. 13)}$$

which upon integration gives

$$W_o^{1/2} - W_t^{1/2} = k''N^{1/2}t \quad \text{(Eq. 14)}$$

where

$$k'' = \frac{3k'\alpha_v^{1/6}DC_s}{2k}$$

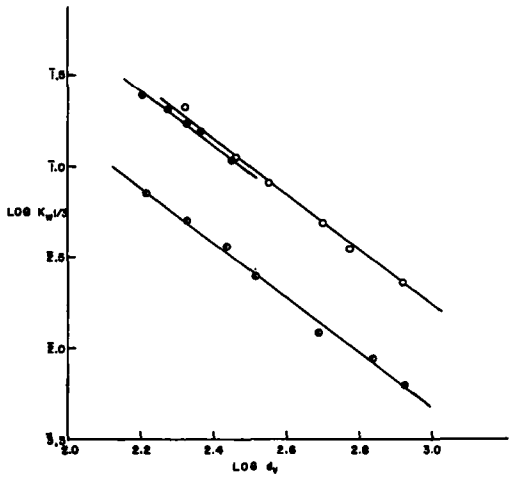

Fig. 3.—Dependency of log initial Hixon-Crowell rate constants on log initial mean volume diameter. Lines have slope of −1.5. —O—O— potassium ferricyanide, 23.4°; —●—● spray-congealed salicylamide, 24.8°; —◐—◐— potassium dichromate, 23.4°.

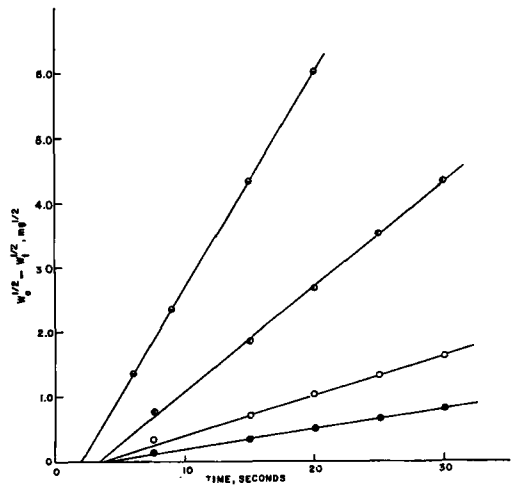

Fig. 4.—Typical dissolution data for potassium dichromate plotted according to Eq. 14. T = 23.4°. ◒—164μ, ◐—272μ, O—489μ, ●—846μ.

All of the data were replotted as $\Delta W^{1/2}$ *vs.* t according to Eq. 14 and were found to be described by this equation even better than by Eq. 4. Figure 4 shows typical data plots of Eq. 14. These rate constants are listed in Table I as $K_w^{1/2}$.

Figure 5 is a model plot of fraction initial diameter against time calculated from Eqs. 4 and 14. It is seen from Fig. 5 that appreciable deviation from the cube root law is not encountered until dissolution has progressed to a region where $d_t \sim 0.6\,d_v$ or 80% of the particle has dissolved. This explains why Eq. 4 described the data within a run but did not correlate data between runs of sufficiently different size material.

As additional tests of Eq. 14, two experiments were run. From Eq. 14 it may be seen that the rate constant depends on the square root of the total number of particles. Since N is proportional to weight, aqueous dissolution rate constants were determined for different initial weights of a single size fraction of crystalline salicylamide (needle habit). Figure 6 shows a plot of $k_w^{1/2}$ against $W_o^{1/2}$ and shows by its linearity that Eq. 14 is valid.

The number of particles can be kept constant for different size fractions by calculating equivalent weights from the size data. When this is done the rate constant given by Eq. 14 should be independent of size while the constant given by Eq. 4 would be independent of size only if δ were constant. Table II gives data obtained from dissolution runs on samples of potassium dichromate in which the number of particles was kept constant.

It can be seen from the data in Table II that $k_w^{1/2}$ is constant but that $k_w^{1/3}$ increases as particle size is decreased. The most exacting test of the experimental method and Eq. 14 would be their ability to describe the complete dissolution curve. One run was made using 0.1000 Gm. of 140–200 mesh potassium dichromate in the previous experiment. In this system the particles completely dissolved to give a solution which could still be assayed directly in the instrument. Figure 7 compares the experimental data using the Hixon-Crowell $\Delta W^{1/3}$ plot and the $\Delta W^{1/2}$ plot derived above.

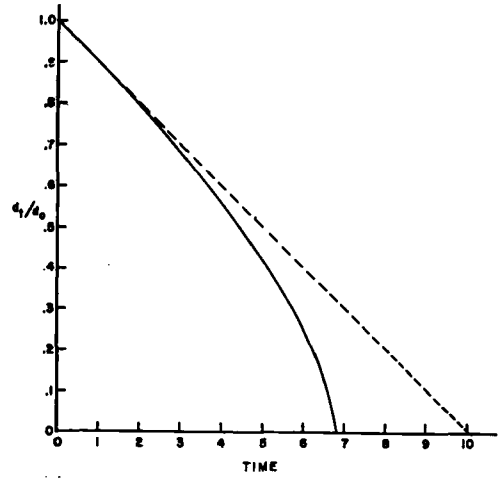

Fig. 5.—Hypothetical plot of the ratio of diameter at time, t, to initial diameter *vs.* time. ——— calculated from Eq. 4. – – – – calculated from Eq. 14.

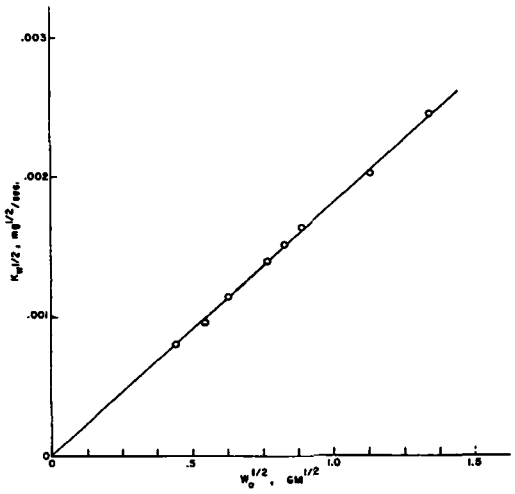

Fig. 6.—Dependency of the rate constant from Eq. 14 on the square root of the initial weight of salicylamide needles. Dissolution in water at 23.4°.

TABLE II.—DISSOLUTION RATE CONSTANTS FROM EQUAL NUMBERS OF POTASSIUM DICHROMATE PARTICLES OF DIFFERENT SIZES[a]

d_v, μ	W_o, Gm.	$K_w^{1/3}$, mg.$^{1/3}$/sec.	$K_w^{1/2}$, mg.$^{1/2}$/sec.
846	13.6292	0.017	0.13
686	7.8053	0.021	0.14
489	2.6407	0.024	0.13
326	0.7810	0.027	0.13
272	0.4560	0.032	0.13
213	0.2181	0.042	0.13
164	0.1000	0.044	0.12

[a] At 25°.

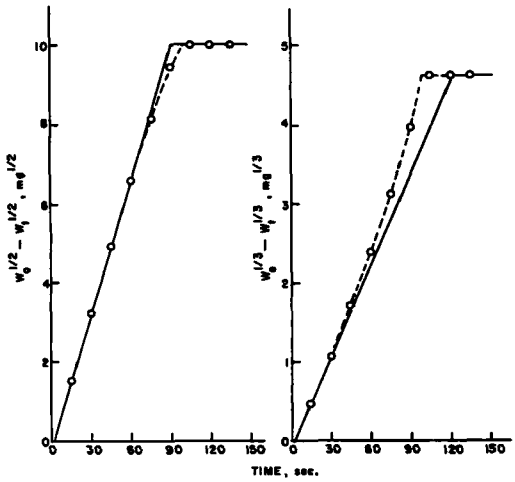

Fig. 7.—Comparison of the ability of Eqs. 14 and 4 to describe the complete dissolution curve for 164μ potassium dichromate. T = 25.0°. – – – – experimental line, ——— line calculated from data obtained in the first 30 seconds.

As seen in Fig. 7 neither equation exactly described the data over the complete run. While the results from the $\Delta W^{1/3}$ plot cannot be explained at this time unless the δ dependency is accepted, there is a valid reason for the deviation in the $\Delta W^{1/2}$ plot. At the point where deviation first occurs, 96% of the material has dissolved and it is not unreasonable to expect that the smaller particles are disappearing. Thus, N would no longer be constant and the curve would be expected to fall off as shown. It was also of interest to note in this run that the final three data points showed that exactly 0.100 Gm. of potassium dichromate had dissolved. This indicates the accuracy of dissolution data obtained with the apparatus.

DISCUSSION

The data obtained in this study clearly show that the cube root law does not describe dissolution of particles under the degree of agitation used. The data were described by an equation derived by making the assumption that δ was proportional to $d_v^{1/2}$. It might be expected that such a simple relationship between δ and d_v should be derivable on a theoretical basis. This type of mathematical treatment has been well developed for flow past a flat surface, but it is generally conceded that flow past a sphere is so complicated that mathematical handling is impossible (15). Solving the Prandtl boundary layer equations for flow past a flat surface result in the relationship (16)

$$\delta = k \frac{L^{1/2}}{v^{1/2}} \qquad \text{(Eq. 15)}$$

where L = the length of the surface in the direction of flow, v = the approach velocity of the flowing liquid. Thus, in the system studied one might expect that the larger particles would be subjected to greater flow velocities as postulated by McCabe and Stevens, but, on the other hand, that their surface dimensions would be larger. These two effects are in opposition as seen from Eq. 15. Certainly, direct measurement of the diffusion layer and correlation of factors affecting it would contribute significantly to the complete understanding of particle dissolution. Until such information is available the possible nonconstancy of δ as dissolution proceeds must be considered in the treatment of particle dissolution data.

SUMMARY

1. Dissolution data for different size fractions of three different compounds were obtained.

2. The data were described by an equation derived on the assumption that the thickness of the diffusion layer was proportional to the square root of the mean volume diameter.

3. Experiments designed to test the equation showed that it was valid under the conditions of study.

4. The applicability of the method of Niebergall and Goyan to dissolution studies was demonstrated.

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Stability of Antibacterial Preservatives in Parenteral Solutions III

Relationship Between Chemical Loss and Microbiological Activity in Multiple-Dose Vials

By L. LACHMAN, S. WEINSTEIN, T. URBANYI, E. EBERSOLD, and J. COOPER

The loss of preservative content due to degradation in solution and absorption by rubber stoppers was studied by chemical and microbiological analytical methods. The preservatives investigated were chlorobutanol and *p*-chloro-β-phenylethyl alcohol in conjunction with neoprene rubber stoppers. The contribution of rubber extractives and preservative degradation products towards enhanced antimicrobial activity is discussed.

THE LOSS of preservatives from solution due to degradation or absorption by rubber stoppers has been determined by chemical (1–4) and microbiological (4–6) methods. However, there are no reports in the literature quantitatively comparing the loss of preservative as determined chemically and microbiologically. This lack of information can be ascribed to two major factors: (*a*) the time consuming and cumbersome characteristics of microbiological procedures and (*b*) the fact that existing microbiological assay methods have not been of sufficient accuracy or precision to permit valid comparisons.

In a previous investigation (7) a turbidimetric microbiological assay method was described for chlorobutanol and *p*-chloro-β-phenylethyl alcohol. This would now permit a valid correlation between chemical and microbiological determinations of preservative content.

Because of the techniques employed in the manufacture of rubber stock to be used for molding rubber stoppers, it is not uncommon for the rubber to contain appreciable amounts of unreacted accelerators, activators, and antioxidants. It has been recognized that these unreacted materials, as well as certain reaction products (8–12), can be extracted by solutions coming in contact with the stopper. These materials have been shown to exert deleterious effects on active ingredients (13) and antibacterial agents (10) in injectable solutions.

In this study the influence of rubber closure extractives on the microbiological activity of the preservatives chlorobutanol and *p*-chloro-β-phenylethyl alcohol in vial solutions buffered to a pH 4 and stoppered with neoprene closures was determined. In addition, the contribution of preservative degradation products to antibacterial activity was ascertained. These two preservatives were chosen for study because chlorobutanol is representative of one which degrades in solution as well as being absorbed by the closure, whereas *p*-chloro-β-phenylethyl alcohol is representative of a preservative which essentially undergoes no degradation for the duration of study but which is lost from solution by absorption into the closure. Analyses for residual preservative content were performed both chemically and microbiologically and the results compared to determine whether rubber extractives or preservative degradation products influence the microbiological determinations.

EXPERIMENTAL

Materials.—0.275 *M* solution of citric acid–sodium phosphate buffer of pH 4.0; *p*-chloro-β-phenylethyl alcohol, Ciba, b.p. 80–83° at 1.07 mm; chlorobutanol, anhydrous U.S.P.; neoprene poly-

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Stability of Antibacterial Preservatives in Parenteral Solutions III

Relationship Between Chemical Loss and Microbiological Activity in Multiple-Dose Vials

By L. LACHMAN, S. WEINSTEIN, T. URBANYI, E. EBERSOLD, and J. COOPER

The loss of preservative content due to degradation in solution and absorption by rubber stoppers was studied by chemical and microbiological analytical methods. The preservatives investigated were chlorobutanol and *p*-chloro-β-phenylethyl alcohol in conjunction with neoprene rubber stoppers. The contribution of rubber extractives and preservative degradation products towards enhanced antimicrobial activity is discussed.

THE LOSS of preservatives from solution due to degradation or absorption by rubber stoppers has been determined by chemical (1–4) and microbiological (4–6) methods. However, there are no reports in the literature quantitatively comparing the loss of preservative as determined chemically and microbiologically. This lack of information can be ascribed to two major factors: (*a*) the time consuming and cumbersome characteristics of microbiological procedures and (*b*) the fact that existing microbiological assay methods have not been of sufficient accuracy or precision to permit valid comparisons.

In a previous investigation (7) a turbidimetric microbiological assay method was described for chlorobutanol and *p*-chloro-β-phenylethyl alcohol. This would now permit a valid correlation between chemical and microbiological determinations of preservative content.

Because of the techniques employed in the manufacture of rubber stock to be used for molding rubber stoppers, it is not uncommon for the rubber to contain appreciable amounts of unreacted accelerators, activators, and antioxidants. It has been recognized that these unreacted materials, as well as certain reaction products (8–12), can be extracted by solutions coming in contact with the stopper. These materials have been shown to exert deleterious effects on active ingredients (13) and antibacterial agents (10) in injectable solutions.

In this study the influence of rubber closure extractives on the microbiological activity of the preservatives chlorobutanol and *p*-chloro-β-phenylethyl alcohol in vial solutions buffered to a pH 4 and stoppered with neoprene closures was determined. In addition, the contribution of preservative degradation products to antibacterial activity was ascertained. These two preservatives were chosen for study because chlorobutanol is representative of one which degrades in solution as well as being absorbed by the closure, whereas *p*-chloro-β-phenylethyl alcohol is representative of a preservative which essentially undergoes no degradation for the duration of study but which is lost from solution by absorption into the closure. Analyses for residual preservative content were performed both chemically and microbiologically and the results compared to determine whether rubber extractives or preservative degradation products influence the microbiological determinations.

EXPERIMENTAL

Materials.—0.275 *M* solution of citric acid–sodium phosphate buffer of pH 4.0; *p*-chloro-β-phenylethyl alcohol, Ciba, b.p. 80–83° at 1.07 mm; chlorobutanol, anhydrous U.S.P.; neoprene poly-

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mer closure No. 704, West Co.; U.S.P. type I, 10-ml. amber ampuls and vials, Kimble Glass Co.; and three-piece aluminum caps for vials Nos. 13–30, West Co.

Equipment.—Bausch & Lomb Spectronic 20 colorimeter; Beckman spectrophotometer model DU; Beckman pH meter model G; and Cary recording spectrophotometer model No. 11.

Preparation of Ampuls and Vials of Preservative Solution.—The rubber closures, vials, and ampuls used in this study were prepared in accordance with the procedures described in a previous publication of this series (14). Solutions of 0.3% *p*-chloro-*β*-phenylethyl alcohol and 0.5% chlorobutanol were prepared on a w/v basis with water for injection buffered to a pH of 4.0. The preservative solutions were then filtered through medium porosity sintered-glass filters. Each preservative solution was filled into 10-ml. amber ampuls and vials. The ampuls were closed by normal pull sealing techniques under an oxygen-gas flame. The vials of each preservative solution were stoppered with the neoprene closures. The stoppered vials were then sealed at a constant head pressure of 60 p.s.i. with a Westcapper. The preservative solutions in ampuls and vials were placed into a constant temperature oven regulated at 60° ± 1.5°. Half of the vials were stored in an upright position and half inverted. At prescribed time intervals, samples were withdrawn and tested chemically for residual preservative content, and microbiologically for residual antibacterial activity.

Analytical Methods.—*Chemical:* The assay methods employed for chlorobutanol and *p*-chloro-*β*-phenylethyl alcohol have been described in an earlier publication of this series (14).

Microbiological: A complete description of the method used for quantitatively determining the residual antibacterial activity of chlorobutanol and *p*-chloro-*β*-phenylethyl alcohol has been presented in the previous paper of this series (7).

RESULTS AND DISCUSSION

The loss of preservative content and its related antibacterial activity was determined by chemical and microbiological methods. Converting the antibacterial activity to preservative concentration permitted a comparison with the residual preservative content measured chemically. This then enabled a valid evaluation of the influence of closure extractives and preservative degradation on antibacterial activity.

TABLE I.—NEOPRENE CLOSURE COMPOSITION AND PHYSICAL PROPERTIES

Composition	Physical Properties
Neoprene polymer[a]	Specific gravity = 1.490
Sulfonated oil	Thickness[b] = 0.125 inch
Calcined clay	Weight[c] = 548 mg.
Barium sulfate	
Zinc oxide	
Iron oxide	
Stearic acid	
Mineral oil	
Imidazoline type accelerator	

[a] Rubber content is 42.4% of total composition. [b] Measured microscopically, and the value is an average of five measurements from different stoppers. [c] Value is an average of ten stoppers.

TABLE II.—PER CENT *p*-CHLORO-*β*-PHENYLETHYL ALCOHOL ABSORBED BY NEOPRENE CLOSURE AT 60°C.

Time, Days	Storage	Chemical Assay	Microbiological Assay
1	Vials upright	3.6	...
	Vials inverted	11.1	...
2	Vials upright	10.6	0
	Vials inverted	14.3	3.7
8	Vials upright	17.9	7.15
	Vials inverted	25.0	17.9
14	Vials upright	25.0	14.3
	Vials inverted	32.1	25.0
21	Vials upright	28.6	21.1
	Vials inverted	35.7	32.1
23	Vials upright	28.6	21.1
	Vials inverted	35.7	32.1
30	Vials upright	28.6	21.1
	Vials inverted	35.7	32.1

The neoprene closures were chosen for this study as a result of earlier data (14) showing that these preservatives exhibited the greatest loss from solution when in contact with these closures. The composition, per cent rubber content, and the physical properties of these closures are given in Table I.

Chemical and Microbiological Results

***p*-Chloro-*β*-phenylethyl Alcohol.**—The data summarized in Table II show the amount of *p*-chloro-*β*-phenylethyl alcohol absorbed by the rubber when the vials are stored at 60° in inverted and upright positions. When plotting these data rectilinearly, as shown in Fig. 1, it is evident from these graphs that at about 21 days the curves develop a plateau. This would indicate that the time required for the absorption to reach an equilibrium concentration is the same for the vials stored in an inverted or upright position. However, the equilibrium concentration of the preservative in the closure is higher for the vials stored in the inverted position as compared with those stored upright.

From the equilibrium portions of the curves it is apparent that the per cent preservative absorbed as determined microbiologically is less than that found chemically. This difference can be attributed to extractives being leached from the rubber stopper and exhibiting antimicrobial activity. When the preservative solutions are assayed microbiologically, the antibacterial activity found is a measure of the biological activity of the residual preservative plus that due to extractives. Therefore, when the microbiological assay data are converted from activity to concentration, it would suggest that less preservative was absorbed. Berry (10) has shown that the presence of tetramethylthiuram disulfide used as an accelerator in rubber compositions exerts a bactericidal effect. The present investigation indicates that the accelerator contained in the neoprene closures, namely 2-mercaptoimidazoline ethylene thiourea, also possesses antibacterial activity.

Since the total concentration of preservative in solution would be expected to be significantly greater than that in the vapor above the solution, there should occur less preservative absorption when the vials are stored upright. That this is the case is shown by comparing the difference between the equilibrium absorption concentrations for the vials stored upright and inverted. As measured

chemically, the difference is about 7% as compared to 11% when measured microbiologically. The 4% difference that exists between these two assay methods must be attributed to extractives being leached from the closure and exerting antibacterial activity. This is confirmed by comparing the equilibrium concentration of preservative absorbed for the vials stored in an inverted position and determined both chemically and microbiologically. The microbiological assay shows about 4% less preservative absorbed when compared with the chemical assay.

Chlorobutanol.—A comparison between chemical and microbiological assays measuring chlorobutanol loss in vial solutions stoppered with neoprene closures and stored at 60° in inverted and upright positions is presented in Table III. It is evident from the degradation data obtained for the ampul solutions that the microbiological assay does not indicate any loss of preservative until there is about a 6% reduction in concentration as measured chemically. It is postulated that this difference is due to the degradation products exerting antibacterial activity. These degradation products were identified by Nair and Lach (15) to be acetone, carbon monoxide, chloride ion, and alpha-hydroxybutyric acid.

It would appear from the data for the vial solutions that the difference between the residual concentration of preservative measured chemically and microbiologically can be mainly ascribed to the antimicrobial activity of the rubber extractives leached from the rubber and to a lesser degree to the antibacterial activity of the degradation products of the preservative.

TABLE III.—PER CENT RESIDUAL CHLOROBUTANOL AS MEASURED CHEMICALLY AND MICROBIOLOGICALLY AFTER STORAGE AT 60°C.

Time, Days	Storage	Chemical Assay	Microbiological Assay
2	Ampul	100	100
	Vials upright	85.6	86.4
	Vials inverted	83.6	88.4
8	Ampul	97.0	100
	Vials upright	69.4	88.4
	Vials inverted	67.4	84.3
16	Ampul	94.5	100
	Vials upright	67.4	78.5
	Vials inverted	63.2	74.5
23	Ampul	92.0	94.0
	Vials upright	65.3	76.5
	Vials inverted	63.2	72.5
30	Ampul	89.4	92.0
	Vials upright	59.2	68.5
	Vials inverted	53.0	68.5

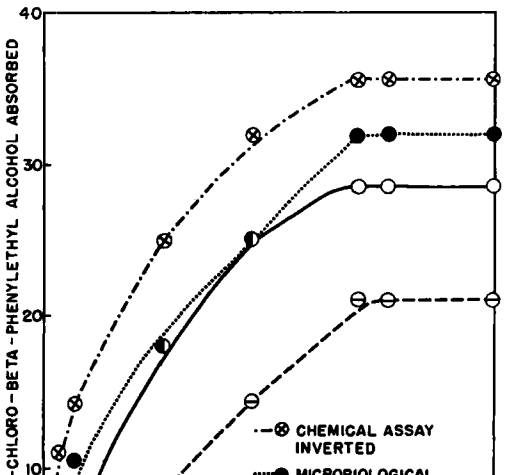

Fig. 1.—The per cent of *p*-chloro-β-phenylethyl alcohol absorbed by the neoprene rubber stoppers from vial solutions stored upright and inverted at 60° as determined chemically and microbiologically.

SUMMARY

This study was undertaken in an effort to determine whether chemical analysis for residual preservative in vial solutions stoppered with rubber closures is a true indication of residual preservative activity. The preservatives evaluated were chlorobutanol which degrades in solution and is absorbed by the rubber and *p*-chloro-β-phenylethyl alcohol which is apparently lost from solution entirely by absorption into the rubber stopper. The data may be summarized as follows:

1. The extractives leached from the neoprene stoppers were found to exhibit demonstrable antimicrobial activity causing the microbiological analysis for residual preservative to be higher than that determined chemically.

2. Although the degradation products of chlorobutanol showed some antibacterial activity, it was substantially less than the extractives leached from the rubber closure.

3. In order to properly determine the influence of rubber closures on preservatives in parenteral solutions, it is important to study the preservative systems both chemically and microbiologically.

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Stability of Antibacterial Preservatives in Parenteral Solutions IV

Contribution of Rubber Closure Composition on Preservative Loss

By LEON LACHMAN, TIBOR URBANYI, and SEYMOUR WEINSTEIN

The loss of benzyl alcohol, phenylethyl alcohol, and methylparaben from solution in the presence of natural, neoprene, and butyl rubber stoppers was studied. The influence of closure extractives on the analysis for residual preservative was evaluated. To insure valid determinations of preservative loss, techniques for eliminating the interference of extractives in the analysis were investigated. Partition studies were performed on the preservatives to determine their relative preference for each rubber composition studied.

THE USE of rubber closures for parenteral solutions in multiple-dose vials has resulted in many compatability problems (1–3). This can be attributed to the complexity of most rubber closure formulations which contain about 40–50% rubber, plus at least seven other components (4). These include vulcanizing agents, activators, accelerators, fillers, pigments, antioxidants, and softeners, all of which are reported to be essential if the stoppers are to possess the desirable physical and chemical properties.

In order to insure complete vulcanization of various type rubbers, excess quantities of accelerators, activators, and other additives are generally used. It has been shown that some of these unreacted agents and reaction products of the vulcanization process can be leached from the closure by parenteral solutions. These materials exert toxic and pyrogenic effects (5, 6) as well as deleterious effects on the stability of the injectable solution (7–10).

In previous investigations conducted at this laboratory (11, 12), rubber closures and rubber extractives were found to significantly influence preservative loss from solution and antimicrobial activity, respectively. In this study an attempt was made to determine (*a*) the effect of neoprene, natural, and butyl rubber closures on the loss of benzyl alcohol, methylparaben, and phenylethyl alcohol from vial solutions, (*b*) the manner in which rubber extractives interfere with the assay methods for residual preservative, and (*c*) the distribution tendencies of the above three preservatives between water buffered to a pH of 4 and each composition rubber stopper.

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EXPERIMENTAL

Materials.—Benzyl alcohol, reagent grade, Fisher Scientific Co., methylparaben, U.S.P.; phenylethyl alcohol, Eastman Organic Chemicals; heptaldehyde aniline reaction product, tetramethyl thiuram monosulfide, and zinc dimethyl dithiocarbamate, West Co.; 0.275 *M* citric acid–sodium phosphate buffer of pH 4.0; natural crepe, neoprene polymer, and butyl polymer rubber stoppers, West Co.; 10-ml. amber ampuls and vials, U. S. P. type I; and three-piece aluminum caps for vials, West Co., No. 13–30.

Equipment.—Beckman spectrophotometer model DU; Cary recording spectrophotometer model No. 11; and Beckman pH meter model G.

Preparation of Ampuls and Vials of Preservative Solutions.—The ampuls, vials, and stoppers were washed in a manner described in a previous study (11). Solutions of 1.0% benzyl alcohol, 0.2% methylparaben, and 0.5% phenylethyl alcohol were prepared on a w/v basis with water for injection buffered to a pH of 4.0. The solutions were filtered through a medium porosity sintered-glass filter. Each preservative solution was filled into 10-ml. ampuls and vials. The ampuls were closed by customary pull sealing procedures with an oxygen-gas flame. The vials of each preservative solution were stoppered with three different composition rubber closures and then sealed with three-piece aluminum caps at a constant sealing head pressure of 60 p.s.i. with a Westcapper. The preservative solutions in ampuls and vials were placed in constant temperature ovens regulated at 25, 40, 50, and 60° ± 1.5°. Half of the vials were stored upright and half inverted. At designated time intervals, samples were withdrawn and tested for residual preservative content, pH, and physical change.

Apparent Distribution of Preservative Between Buffer Solution and Rubber.—The procedure employed has been described in detail in a previous publication (11). This evaluation was done for the three stoppers: neoprene, natural, and butyl rubber, and the preservatives: benzyl alcohol, phenylethyl alcohol, and methylparaben.

Analytical Methods

Benzyl Alcohol.—The concentration of preserva-

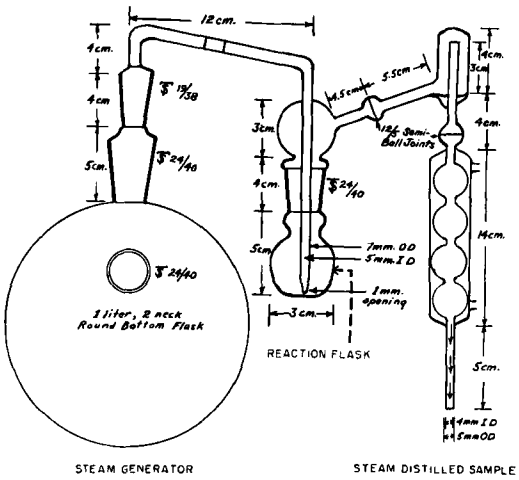

Fig. 1.—Microsteam-distillation apparatus.

tive was determined by pipetting 1 ml. of solution into a microsteam-distillation apparatus (Fig. 1) and the sample distilled to 25 ml. Absorbance was measured at 257 mμ where the $A(1\%, 1\text{ cm.}) = 18$.

Phenylethyl Alcohol.—The concentration of preservative was determined by pipetting 3 ml. of solution into a microsteam-distillation apparatus and the sample steam distilled to 30 ml. The distillate was brought up to volume in a 50-ml. volumetric flask with distilled water. Absorbance was measured at 257 mμ where $A(1\%, 1\text{ cm.}) = 18$.

Methylparaben.—A 3-ml. aliquot was pipetted into a 50-ml. volumetric flask and brought up to volume with methanol. Five milliliters of this solution was transferred to a 100-ml. volumetric flask and brought up to volume with methanol. Absorbance was measured at 256 mμ where $A(1\%, 1\text{ cm.}) = 1115$.

TABLE I.—BUTYL RUBBER COMPOSITION AND PHYSICAL PROPERTIES

Composition	Physical Properties
Butyl polymer[a]	Specific Gravity = 1.609
Barium sulfate	Thickness[b] = 0.125 inches
Calcined clay	Weight[c] = 597 mg.
Carbon black	
Titanium dioxide	
Zinc oxide	
Stearic acid	
Paraffin wax	
Sulfur	
Thiuram combined with aniline reaction product	

[a] Rubber content is 36.8% of total composition. [b] Measured microscopically, and the value is an average of five measurements from different stoppers. [c] Value is an average of ten stoppers.

RESULTS AND DISCUSSION

The stability of several bacteriological preservatives in aqueous buffered solutions stored in multiple-dose vials was studied. The rubber stoppers used in this investigation are representative of three closure compositions frequently employed for multiple-dose vials. The composition, per cent rubber, and physical properties for the neoprene and natural rubber stoppers were presented in a previous paper (11). However, a different butyl stopper was used in place of the one used earlier because there was an obvious physical incompatability with the preservative solutions used. The composition and physical properties for this closure are presented in Table I.

Benzyl Alcohol

The influence of neoprene, natural, and butyl rubber stoppers on the stability of benzyl alcohol in aqueous solution buffered to a pH of 4.0 was investigated. The results obtained from ampul and vial solutions are summarized in Table II. It is evident from the data in this table that for the vials stoppered with butyl rubber closures, there appears to be a substantial increase in benzyl alcohol concentration with time instead of the expected loss or maintenance of concentration. This also seems to occur for the vial solutions stoppered with neoprene closures, but to a lesser degree. However, for the vials stoppered with the natural rubber closures, this effect was not observed.

As a result of this unexpected finding, investigations were initiated to determine whether extractives from the rubber stopper were responsible for the apparent increase in benzyl alcohol content. After reviewing the composition of the butyl rubber closure (Table I), it was presumed that the presence of unreacted accelerators or their reaction products would most likely be the causative agents for the difficulties being encountered. If these agents were leached from the closure by the benzyl alcohol solution they could interfere with the analysis for residual preservative content by exhibiting high absorptivity in the wavelength region of benzyl alcohol.

In order to verify this assumption, samples of the primary (heptaldehyde aniline reaction product) and secondary (tetramethyl thiuram monosulfide) accelerators and the reaction product of the accelerators (zinc dimethyl dithiocarbamate) were obtained from the rubber closure manufacturer and analyzed according to the procedure used for the preservative solution. For the heptaldehyde aniline reaction product, 1 ml. was steam distilled in a manner similar to that used for benzyl alcohol, while for the secondary accelerator and the reaction product of the accelerators, separate saturated solutions were prepared in pH 4 buffer and a 3-ml.

TABLE II.—PER CENT RESIDUAL BENZYL ALCOHOL AFTER STORAGE AT 60° C.

Time, weeks	Ampul	Butyl Rubber		Neoprene Rubber		Natural Rubber	
		Upright	Inverted	Upright	Inverted	Upright	Inverted
2	100	105	110	96	98	96	98
4	100	106	124	96	97	95	95
8	102	115	127	99	107	89	92
12	103	124	131	102	102	87	88

aliquot removed and steam distilled. The absorption curves in the ultraviolet region for the distilled samples are shown in Fig. 2. It is evident from these curves that a volatile component of the heptaldehyde aniline reaction product exhibits strong absorption in the region of 257 mμ, the wavelength where measurements are made for benzyl alcohol. The other two components, namely tetramethyl thiuram monosulfide and zinc dimethyl dithiocarbamate, show very low absorption at 280 mμ and no absorption at 257 mμ.

The influence of the closure extractives on the absorption characteristics of benzyl alcohol stored as a buffered solution in vials stoppered with the butyl rubber closure is illustrated in Fig. 3. By studying the curve for the zero time sample, it is apparent that the absorbance values for the minimum at 253 mμ and the maximum at 263 mμ are equal.

However, the curve for the sample stored for 12 weeks at 60° shows a large difference between the absorbance at 253 mμ and 263 mμ. This difference can be ascribed to the influence of the absorption characteristic of the volatile component of the primary accelerator which is leached into the benzyl alcohol solution from the rubber stopper. The effect at 253 mμ and 263 mμ causes a concurrent effect at 257 mμ, the absorption maximum used for benzyl alcohol analysis.

In order to correct the absorption readings for benzyl alcohol to deduct the amount due to extractives, two procedures were evaluated.

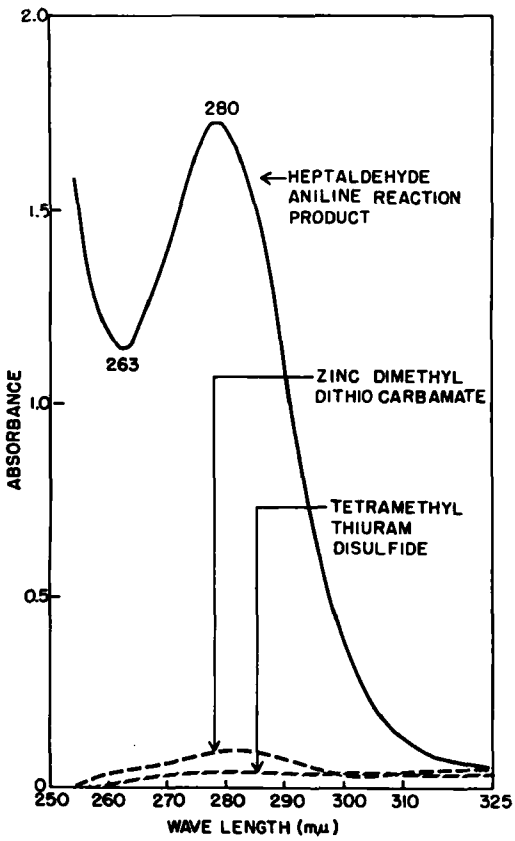

Fig. 2.—The ultraviolet absorption spectra of the accelerators and a reaction product found in the butyl rubber stoppers.

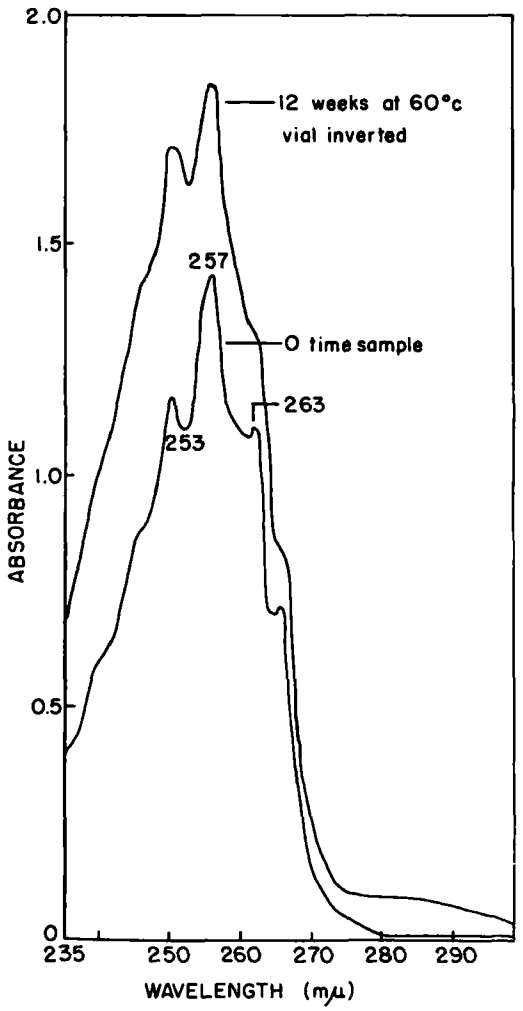

Fig. 3.—Plots showing the influence of butyl rubber closure extractives on the absorption characteristics of benzyl alcohol.

Method I.—The following equation was developed to correct for the absorbance due to extractive and thus give the true absorbance at 257 mμ for benzyl alcohol: $A = B - (C - D)$ where A = corrected absorbance at 257 mμ; B = observed absorbance at 257 mμ; C = observed absorbance at 253 mμ; D = observed absorbance at 263 mμ.

Using this equation, the uncorrected data in Table II for the vials stoppered with butyl rubber stoppers were recalculated and are presented in Table III. It is evident from this table that the corrected values are still higher than the original concentration in most instances. However, if the data are now further corrected for benzaldehyde development measured at 280 mμ, the results presented in the last two columns of Table III are obtained. These data now show that no absorption of benzyl alcohol by the butyl rubber stopper results when the vials are stored in an inverted or upright position.

Method II.—An alternate method used to correct

TABLE III.—CORRECTED PER CENT RESIDUAL BENZYL ALCOHOL IN VIALS STOPPERED WITH BUTYL RUBBER AFTER STORAGE AT 60° C.

Time, weeks	Uncorrected		Corrected			
			Extractives		Extractives and Benzaldehyde	
	Upright	Inverted	Upright	Inverted	Upright	Inverted
2	105	110	100	101	100	100
4	106	124	101	105	99	101
8	115	127	105	107	101	100
12	124	131	103	108	100	102

for the absorbance at 257 mμ due to extractives was a modification of the Morton-Stubb technique (13). A model is presented in Fig. 4 representing pure benzyl alcohol and benzyl alcohol containing extractives which influence the characteristics of the absorption curve. Points *A* and *B* in Fig. 4 are representative of the equal absorbance at the minimum of 253 mμ and maximum of 263 mμ shown in Fig. 3 for pure benzyl alcohol. These wavelengths in the model are represented by λ_2 and λ_3. The wavelength of 257 mμ used in the analysis for benzyl alcohol is represented in the model by λ_1. Using the curves in Fig. 4 as simplified models of the benzyl alcohol absorption curves shown in Fig. 3, an equation was derived which corrects for the absorbance due to extractives.

CE = increase in absorbance at 257 mμ due to extractives; $CD = \frac{DG}{FG} \times HF$ by trigonometry; where HF represents the difference between the observed absorbance at λ_2 and λ_3. $DG = \lambda_3 - \lambda_1$ and $FG = \lambda_3 - \lambda_2$.

If DE is denoted by X, then the corrected value for the absorbance at λ_1 is $A\lambda_1$ corrected $= A\lambda_1$ observed $- CD - X$ and at λ_2 is $A\lambda_2$ corrected $= A\lambda_3$ observed $- X$. Therefore, the ratio of these must be equal to the known ratio for the pure substance designated as K

$$K = \frac{A\lambda_1 \text{ observed} - CD - X}{A\lambda_3 \text{ observed} - X} = 1.31$$

for pure benzyl alcohol.

Since DG = 263 mμ − 257 mμ = 6 mμ and FG = 263 mμ − 253 mμ = 10 mμ then $\frac{DG}{FG} = 0.6$

$$K = \frac{A^{1\%}_{257} - (A^{1\%}_{253} - A^{1\%}_{263})\,0.6 - X}{A^{1\%}_{263} - X}$$

Solving for X

$$A^{1\%}_{257} - (A^{1\%}_{253} - A^{1\%}_{263})\,0.6 - X =$$
$$(A^{1\%}_{263} - X)\,1.31 - X + 1.31\,X =$$
$$[(A^{1\%}_{263})\,1.31] - [A^{1\%}_{257} - (A^{1\%}_{253} - A^{1\%}_{263})\,0.6]$$
$$X = \frac{[(A^{1\%}_{263})\,1.31] - [A^{1\%}_{257} - (A^{1\%}_{253} - A^{1\%}_{263})\,0.6]}{0.31}$$
$$CD = (A^{1\%}_{253} - A^{1\%}_{263})\,0.6$$
$$A^{1\%}_{257} \text{ corrected} = A^{1\%}_{257} \text{ observed} - (X + CD)$$

The data for the vials stoppered with the butyl rubber closures were corrected according to this equation and the corrected data were found to be in agreement with the data obtained from the equation in Method I. This indicates that either Method I or II satisfactorily corrects for the absorbance due to extractives. However, since Method I is simpler than Method II, it was used to correct the data for the vials stoppered with the neoprene closures.

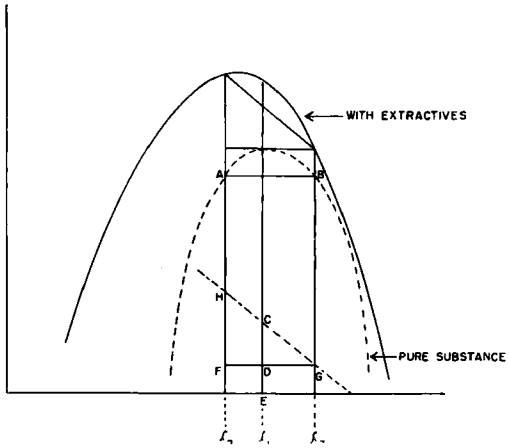

Fig. 4.—Model curves developed from the absorption plots shown in Fig. 3 for use in developing the modified Morton-Stubbs correction for benzyl alcohol assay.

The results in Table IV show the corrected and uncorrected values for the per cent preservative

TABLE IV.—CORRECTED AND UNCORRECTED PER CENT RESIDUAL BENZYL ALCOHOL IN VIALS STOPPERED WITH NEOPRENE RUBBER AFTER STORAGE AT 60°C.

Time, weeks	Uncorrected		Corrected	
	Upright	Inverted	Upright	Inverted
2	96	98	94	95
4	96	97	92	92
8	99	107	90	92
12	102	102	91	90

absorbed by the neoprene rubber stoppers. These stoppers behave differently from the butyl rubber, in that they absorb preservative from solution as well as leach out extractives. This is particularly apparent from the data for the vials stored in an inverted position. The decrease in apparent benzyl alcohol concentration after 12 weeks storage indicates that the benzyl alcohol is continuing to be absorbed by the closure while the amount of extractives being leached from the closure has apparently reached an equilibrium concentration somewhere between 8 and 12 weeks.

In Table V is presented the corrected per cent residual benzyl alcohol for the ampul and vial solutions stoppered with the three closures. It can be seen from this table that the natural rubber

TABLE V.—THE CORRECT PER CENT RESIDUAL BENZYL ALCOHOL AFTER STORAGE AT 60° C.

Time, weeks	Ampul	Butyl Rubber		Neoprene Rubber		Natural Rubber	
		Upright	Inverted	Upright	Inverted	Upright	Inverted
2	100	100	100	94	95	96	98
4	100	99	101	92	92	95	95
8	101	101	100	90	92	89	92
12	100	100	102	91	90	87	88

absorbs slightly more benzyl alcohol than the neoprene rubber. However, it is interesting to note that for both stoppers the amount of preservative absorbed is the same for the vials stored in an upright and inverted position.

From the data presented above, it is apparent that extractives leached from closures may have a significant effect on the assay for residual preservatives. Consequently, when assay results for preservative content appear to be irregular or show an increase with storage, consideration should not only be given to the assay method but also to the possibility that extractives are being leached from the rubber stoppers and interfering with the assay. If the assay method is a spectrophotometric one, it is necessary to run the complete absorption curves in order to adequately demonstrate the interfering characteristics of the extractives.

Methylparaben

The stability of a 0.2% solution of methylparaben (pH 4.0) in vials stoppered with natural, neoprene, and butyl rubber closures and stored at 60° was investigated. According to the data presented in Table VI, methylparaben appears to exhibit good stability in the presence of butyl rubber and fair stability with natural and neoprene rubber, with the latter causing the greater deleterious effect. As in the case of benzyl alcohol, the amount of methylparaben lost from solution was the same in vials stored upright or inverted.

It is interesting to note that although residual methylparaben was assayed spectrophotometrically at 256 mμ which is extremely close to the 257 mμ used for benzyl alcohol, no interference was observed due to extractives. This may be explained as follows: (*a*) since methylparaben is a solid, it does not exert any solublizing effect on the unreacted accelerator in the rubber, (*b*) the steam distilling of the benzyl alcohol solution before assaying could concentrate the volatile component responsible for interfering with the assay for benzyl alcohol, and (*c*) the high $A^{1\%}_{1cm.}$ of 1115 for methylparaben compared to 18 for benzyl alcohol could diminish any effect due to the absorptivity of the extractives.

Phenylethyl Alcohol

The influence of neoprene, natural, and butyl rubber stoppers on the preservative content of a 0.5% aqueous buffered solution of phenylethyl alcohol (pH 4.0) stored in vials at 60° was studied. It became evident early in the study that the solutions in the vials as well as in the ampuls developed a very fine precipitate which remained fairly well distributed throughout the solution. This precipitate was found to interfere with the spectrophotometric assay for residual preservative since it appeared to distill over from the aliquot of phenylethyl alcohol solution taken for assay, causing high assays for phenylethyl alcohol. In fact, after 12 weeks storage at 60°, the ampuls assayed 112% phenylethyl alcohol. Because of the interfering properties of the precipitate on the assay results, it was felt that the data were not adequate for presentation in this report.

It is believed that the precipitate is due to oxidation products of phenylethyl alcohol, namely, phenylacetaldehyde and phenylacetic acid. Further information will be presented on this preservative at a later date when the studies presently underway to elucidate the responsible factors for the precipitate development are completed.

Apparent Distribution of Preservative Between Rubber and Solution.—In an earlier report (11) data were presented on the distribution of *p*-chloro-β-phenylethyl alcohol and phenylethyl alcohol between rubber and water buffered to a pH of 4.0. These tests were performed with neoprene and natural rubber stoppers.

Similar studies were performed in this investigation for the preservatives chlorobutanol, benzyl alcohol, and methylparaben. The apparent partition coefficients for these three preservatives, as well as for the two studied in the earlier investigation, are summarized in Table VII. These data indicate that of the five preservatives, benzyl alcohol exhibits the least tendency to distribute into either the natural or neoprene rubber of the five preservatives tested. For each preservative the neoprene stoppers absorb to a greater degree than the natural rubber.

From the distribution data in this table, the natural rubber stoppers show the following order of absorption of preservative: benzyl alcohol < methylparaben < phenylethyl alcohol < *p*-chloro-β-phenylethyl alcohol < chlorobutanol. The results for the neoprene stoppers are as follows: benzyl alcohol < phenylethyl alcohol < methylparaben < chlorobutanol < *p*-chloro-β-phenylethyl alcohol. It is interesting to note that the order of absorption is not the same for the two composition closures. The preservative being most absorbed by the natural

TABLE VI.—PER CENT RESIDUAL METHYLPARABEN AFTER STORAGE AT 60° C.

Time, weeks	Ampul	Butyl Rubber		Neoprene Rubber		Natural Rubber	
		Upright	Inverted	Upright	Inverted	Upright	Inverted
2	100	100	100	100	100	100	100
4	100	100	100	100	94.5	100	100
8	100	100	100	94.5	94.5	100	94.5
12	100	100	100	89	89	94.5	94.5

TABLE VII.—APPARENT DISTRIBUTION OF PRESERVATIVE BETWEEN RUBBER AND BUFFER SOLUTIONS[a] AFTER FOUR WEEKS STORAGE

$$K_T = \frac{C_R}{C_B}$$

Preservative	Closure	Temperature, °C. 25	40
Phenylethyl alcohol	Natural	1.72	1.39
	Neoprene	4.23	4.13
p-Chloro-β-phenyl-ethyl alcohol	Natural	6.05	5.70
	Neoprene	16.40	21.80
Chlorobutanol	Natural	9.85	6.83
	Neoprene	14.50	14.50
Benzyl alcohol	Natural	0.63	0.63
	Neoprene	1.66	1.93
Methylparaben	Natural	1.36	1.43
	Neoprene	7.27	8.40

[a] Solutions buffered to a pH of 4.0.

rubber is chlorobutanol while for the neoprene rubber it is *p*-chloro-β-phenylethyl alcohol.

It is common practice for rubber closure manufacturers to use an excess of accelerators and additives in rubber closure formulations. As a result, there exists at the completion of the manufacturing process certain unreacted components in the rubber stock. The quantity of unreacted accelerators present in synthetic rubbers would be expected to be greater than in natural rubber. This is due to the fact that the synthetic rubber, such as butyl and neoprene, is generally more inert than natural rubber, thus making the vulcanization process more difficult. In order to minimize this difficulty, higher dosages of accelerator are used (14).

The results obtained in this investigation, as well as those reported by other investigators, show that the unreacted components of the closure could be extracted by the solution in the vials. These extractives could be extremely dangerous in that they can (*a*) affect the toxicity of the injectable solution, (*b*) interact with components in the solution and cause physical and chemical instability, (*c*) influence bactericidal activity, and (*d*) as illustrated in this investigation, seriously interfere with the analysis of the solution. Consequently, it is essential that rubber stopper manufacturers use more precise formulations and adequate quality standards in the manufacture of rubber closures.

SUMMARY

The interaction between several composition rubber stoppers and three preservatives was evaluated. Aqueous buffered solutions of the preservatives benzyl alcohol, methylparaben, and phenylethyl alcohol were filled into multiple-dose vials and stoppered with natural, neoprene, and butyl rubber closures. These vials were then stored at several temperatures and tested at designated time intervals for the effect of closure on preservative content. The data from this study may be summarized as follows:

1. Two methods were developed to correct for the interference of rubber closure extractives on the analytical procedure used for benzyl alcohol.

2. The butyl rubber closures, although leaching extractives into the preservative solutions, exhibited the least absorbing tendencies toward benzyl alcohol and methylparaben.

3. The amount of benzyl alcohol and methylparaben lost from vial solutions was not significantly influenced by storage of the vials in an upright or inverted position.

4. Distribution studies have shown that both the natural and neoprene rubber stoppers absorbed preservative from solution with the latter closure exhibiting greater absorbing tendencies.

5. From the distribution data in this and a previous study, the natural rubber stoppers exhibit the following order of preservative absorption: benzyl alcohol < methylparaben < phenylethyl alcohol < *p*-chloro-β-phenylethyl alcohol < chlorobutanol. For the neoprene stoppers the following order exists: benzyl alcohol < phenylethyl alcohol < methylparaben < chlorobutanol < *p*-chloro-β-phenylethyl alcohol.

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Some 2-Substituted Derivatives of 2-Dimethylamino-1,2,3,4-tetrahydronaphthalene

By WILLIAM H. SHELVER† and ALFRED BURGER

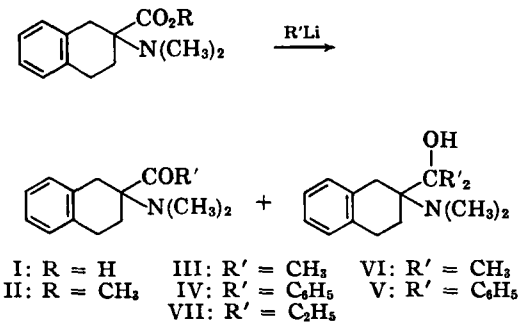
The synthesis and pharmacological examination of several 2-dimethylamino-1,2,3,4-tetrahydronaphthalene 2-alcohols, 2-esters, and 2-ketones is described.

IT HAS BEEN postulated that potent morphine-type analgetics must contain a rigid structure in which the position of the basic nitrogen relative to an aromatic ring is fixed within narrow limits (1). In morphinan the 1,3-fused ring system serves to hold the nitrogen in the axial conformation. Since morphinan contains the 2-amino-1,2,3,4-tetrahydronaphthalene moiety, properly designed derivatives of this structural segment could be candidates for analgetic tests. In simple derivatives of 2-amino-1,2,3,4- tetrahydronaphthalene, the nitrogen will tend to assume the more stable equatorial position, and means other than ring fusion have now been considered to produce axial amino derivatives. Bulky groups in the 2-position with some degree of polarity, whose solvation may further increase their bulk, may cause a dimethylamino group at position 2 to assume an axial conformation. This article describes the synthesis of such compounds. Because bulky groups, on the other hand, may interfere with receptor fit, several compounds containing smaller ester and keto groups in position 2 were also prepared for pharmacological evaluation.

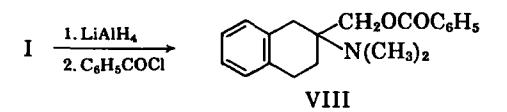
Starting with 2-amino-2-carboxy-1,2,3,4-tetrahydronaphthalene (2), the amino group was methylated by an adaptation of the method of Clarke, *et al.* (3), and the carboxyl function was esterified. The synthesis of alkyl and aryl ketones proceeded from 2-carboxy-2-dimethylamino-1,2,3,4-tetrahydronaphthalene (I), or better from its methyl ester (II). Treatment of I with methyllithium at 25° gave small yields of 2-acetyl-2-dimethylamino-1,2,3,4-tetrahydronaphthalene (III) besides nonbasic decomposition products. Phenyllithium led to a mixture from which 2-benzoyl-2-dimethylamino- (IV) and 2-dimethylamino-2-(α-hydroxybenzhydryl)-1,2,3,4-tetrahydronaphthalene (V) could be separated. Compound V was also obtained from the reaction of phenyllithium and II at 25°, no ketone being isolated in this case. By contrast, the action of methyllithium on II furnished a mixture which on chromatography yielded 55% of 2-acetyl-2-dimethylamino-1,2,3,4-tetrahydronaphthalene (III) and 22% of 2-dimethylamino-2-(2-hydroxy-2-propyl)-1,2,3,4-tetrahydronaphthalene (VI). Reaction of II with ethyllithium furnished a mixture from which only a small amount of 2-dimethylamino-2-propionyl-1,2,3,4-tetrahydronaphthalene (VII) could be elaborated.

Reduction of I with lithium aluminum hydride gave 2-dimethylamino-2-hydroxymethyl-1,2,3,4-tetrahydronaphthalene whose benzoate ester (VIII) was prepared for pharmacological tests.

Compounds II, III, V, and VIII were screened pharmacologically.[1] None of them exhibited analgetic properties in mice at doses up to 200 mg./Kg.; the substances gave no indication of central nervous system depression or excitation, and did not produce hypoglycemia three hours after oral administration of 100 mg./Kg. to 18-hour fasted guinea pigs.



I: R = H III: R′ = CH_3 VI: R′ = CH_3
II: R = CH_3 IV: R′ = C_6H_5 V: R′ = C_6H_5
VII: R′ = C_2H_5



VIII

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[1] By Smith Kline and French Laboratories, to whom the authors are indebted for these tests.

EXPERIMENTAL[2]

2 - Carboxy - 2 - dimethylamino - 1,2,3,4 - tetrahydronaphthalene (I).—A mixture of 2-amino-2-carboxy-1,2,3,4-tetrahydronaphthalene(2)(140 Gm., 0.73 mole), formaldehyde solution U.S.P. (1 L.), and 98% formic acid (1 L.) was refluxed for 10 hours; 100 ml. of concentrated hydrochloric acid was added, and volatile materials were removed by distillation. To the oily residue was added ethanol (750 ml.), and the solid which separated was filtered and washed with ether. Yield, 110 Gm. (70%). Recrystallization from ethanol gave a colorless material, m.p. 223–227° (decompn.). The infrared spectrum was compatible with an amino acid hydrochloride.

Anal.—Calcd. for $C_{13}H_{17}NO_2 \cdot HCl$: C, 61.29; H, 7.12. Found: C, 61.19; H, 6.94.

2 - Carbomethoxy - 2 - dimethylamino - 1,2,3,4-tetrahydronaphthalene (II).—A solution of I (50 Gm., 0.2 mole) in methanol (300 ml.) was treated slowly with a solution of diazomethane, prepared from 110 Gm. of N-methyl-N-nitrosourea, in 2 L. of ether at 0°. After standing overnight, most of the solvents were distilled off, the residue was taken up in ether, washed with potassium hydroxide solution, dried over sodium sulfate, and the ether was evaporated. The residual oil (34 Gm., 71%; b.p. 137°/0.3 mm.; n_D^{25} 1.5310) gave a hydrobromide salt which was recrystallized from isopropyl alcohol-isopropyl ether, m.p. 180°. The infrared spectrum had bands at 3.4, 3.6, 5.8, 6.3, 6.7, 6.9, 7.3, 8–8.5, 9.0, 9.3, 9.7, 9.9, 10.1, 11.0, 11.5, 12.0, 12.6, 12.8, 13.4, 14.2, and 14.8μ.

Anal.—Calcd. for $C_{14}H_{19}NO_2 \cdot HBr$: C, 53.34; H, 6.40. Found: C, 53.54; H, 6.54.

A small yield of the same methyl ester was obtained by refluxing I with equal weights of methanol and sulfuric acid.

2 - Carbethoxy - 2 - diethylamino - 1,2,3,4 -tetrahydronaphthalene.—Prepared in the same manner as the methyl ester, using diazoethane or ethanol and sulfuric acid. Yield 50%, based on weight of oil. Infrared bands of the base were at 3.4, 3.6, 5.8, 6.7, 6.9, 7.3, 8.3, 9.0, 9.75, 10.1, 11.0, 11.4, 12.4, 13.5, and 14.1μ. The hydrobromide, prepared in ether solution, was recrystallized from isopropyl alcohol-isopropyl ether, m.p. 160°.

Anal.—Calcd. for $C_{15}H_{21}NO_2 \cdot HBr$: C, 54.71; H, 6.74. Found: C, 54.44; H, 6.65.

Reaction of I with Phenyllithium.—To a stirred solution of 0.3 mole of phenyllithium in 300 ml. of ether was added, under nitrogen, 10 Gm. (0.04 mole) of I-hydrochloride in quantities of about 0.5 Gm., with sufficient time between additions to allow vigorous refluxing to cease. After 3 hours at 25° the mixture was poured on ice and the ether layer washed with dilute hydrochloric acid to dissolve all basic materials. The acid extracts were made basic with potassium hydroxide solution and extracted with ether. The combined ether extracts were dried and evaporated. The solid residue was crystallized fractionally from ethanol. The more soluble product (18%) melted at 82–83°; its infrared spectrum showed bands at 5.95μ (C=O), and 13.3 and 13.9μ (monosubstituted phenyl); there was no OH band. It represented the phenyl ketone IV.

Anal.—Calcd. for $C_{19}H_{21}NO$: C, 81.68; H, 7.57. Found: C, 81.75; H, 7.25.

The colorless hydrobromide crystallized from isopropyl alcohol-isopropyl ether, m.p. 175–176°.

Anal.—Calcd. for $C_{19}H_{21}NO \cdot HBr$: C, 63.33; H, 6.16. Found: C, 63.37; H, 6.28.

The less soluble compound (28%), m.p. 155–157°, was identical with V and obtained in better yield as described in the next experiment.

2 - Dimethylamino - 2 - (α - hydroxybenzhydryl)-1,2,3,4-tetrahydronaphthalene (V).—To 0.3 mole of phenyllithium in 300 ml. of ether was added 10 Gm. (0.04 mole) of II. The mixture was stirred under nitrogen at 25° for 6 hours, decomposed with ice, and the ether layer was dried over sodium sulfate and evaporated. The solid residue (60% yield) was recrystallized from ethanol, m.p. 155–157°. The material was identical (mixture m.p. and infrared spectra) with the less soluble substance described in the preceding experiment.

Anal.—Calcd. for $C_{25}H_{27}NO$: C, 83.99; H, 7.61. Found: C, 83.58; H, 7.59.

Reaction of 2-Carbomethoxy-2-dimethylamino-1,2,3,4-tetrahydronaphthalene (II) with Methyllithium.—A mixture of 0.3 mole of methyllithium and 10 Gm. of II in 300 ml. of ether was stirred for 3 hours, poured on ice, and the ether layer was extracted well with dilute hydrochloric acid The acid solution was made alkaline and re-extracted with ether. The oily residue from the dried ether extracts exhibited a large C=O band in the infrared, and a small OH band, and the latter did not increase when, in another experiment, the reaction time was extended to 9 hours. Chromatography in ether on Alcoa F-20 alumina furnished 5 Gm. (55%) of a solid, m.p. 52–53°; infrared peaks at 3.4, 3.6, 5.8, 6.75, 6.85, 7.4, 7.9, 8.4, 8.7, 8.9, 9.3, 9.5, 9.9, 10.75, 11.2, 11.9, and 13.3μ. This material represented 2-acetyl-2-dimethylamino-1,2,3,4-tetrahydronaphthalene (III) and was identical with a substance obtained in low yield from I and methyllithium.

Anal.—Calcd. for $C_{14}H_{19}NO$: C, 77.37; H, 8.81. Found: C, 77.04; H, 8.71.

The hydrobromide crystallized from isopropyl alcohol-isopropyl ether, m.p. 173–175°.

Anal.—Calcd. for $C_{14}H_{19}NO \cdot HBr$: C, 56.37; H, 6.75. Found: C, 55.91; H, 6.72.

A second, oily, fraction from the alumina column weighed 2 Gm. (22%) and represented the alcohol VI; infrared bands at 2.8, 3.4, 3.6, 6.3, 6.7, 6.9, 7.3, 7.7, 7.9, 8.1, 8.4, 9.0, 9.3, 9.6, 10.0, 10.6, 10.9, 11.1, 11.5, 11.8, 12.1, 12.4, 13.4, and 14.8 μ.

2 - Dimethylamino - 2 - propionyl - 1,2,3,4 - tetrahydronaphthalene (VIII).—This ethyl ketone was prepared as described for the lower homolog (III), using ethyllithium. The oily reaction product (6 Gm., 58%) exhibited both carbonyl and hydroxyl peaks in the infrared; only after chromatography could a solid salt be obtained from the ketonic fraction. The hydrobromide crystallized from isopropyl alcohol-isopropyl ether, m.p. 161–164°.

Anal.—Calcd. for $C_{15}H_{21}NO \cdot HBr$: C, 57.69; H, 7.10. Found: C, 57.90; H, 6.90.

2 - Benzoyloxymethyl - 2 - dimethylamino - 1,2,3,4-tetrahydronaphthalene (VIII).—To a filtered and stirred solution of 10 Gm. (1.25 moles) of lithium aluminum hydride was added, under nitrogen and in

[2] All melting points are corrected, boiling points uncorrected. Microanalyses by Mrs. Margaret Logan and Mrs. Dorothy Ellis.

small portions, 10 Gm. (0.04 mole) of I-hydrochloride. The mixture was refluxed for 2 hours, allowed to stand at 26° for 6 hours, decomposed with water and worked up as usual. The oily yellow material (6.5 Gm., 82%) had a strong OH peak in its infrared spectrum. It was benzoylated directly with 20 ml. of benzoyl chloride and 150 ml. of 10% sodium hydroxide solution for 15 minutes, the mixture was decomposed with ice, extracted with dilute hydrochloric acid, the acid solutions were made alkaline and re-extracted with ether. The oily product from the dried ether extract weighed 5 Gm. (50%) and exhibited an ester band in its infrared spectrum. Its hydrobromide crystallized from ethyl formate-isopropyl ether, m.p. 194–197°.

Anal.—Calcd. for $C_{20}H_{23}NO_2 \cdot HBr$: C, 61.54; H, 5.94. Found: C, 61.00; H, 6.10.

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Analysis of the Volatile Components of Ylang-Ylang Oil by Gas Chromatography

By DAVID B. KATAGUE and ERNST R. KIRCH

Five samples of commercially available ylang-ylang oil were analyzed by gas chromatography. Of the various stationary liquid phases used, a 20% Ucon on Chromosorb P(w/w) gave consistently the best separation. The composition of extra, first, second, and third quality fractions and pure oil No. 123 was determined on the basis of relative retention times.

Most essential oils are complex mixtures of individual organic compounds that contain varied functional groups which make complete and detailed analysis relatively difficult or at times impossible using conventional procedures.

Thus, prior to the advent of gas chromatography, the analyses of essential oils were tedious and time consuming. Furthermore, in certain instances in which other methods were used, the analyses were not considered complete (1). The availability and development of gas chromatography as an analytical tool has not only greatly facilitated the separation, and at times the identification, of the constituents of a number of essential oils, but also reduced the time required for analyses (2, 3).

One of the important ingredients in a number of perfumes and other cosmetic products is ylang-ylang oil. This is obtained from the flowers of the ylang-ylang tree (*Cananga odorata*, Hook *f et* Thomson), which is probably a native of the Philippines (1). The important commercial sources of this oil for use in the United States today are the Nossi-Be and Comoro Islands. The oil is available in four different quality fractions, based on the respective boiling points, and is classified commercially as extra, first, second, and third quality fractions. The extra fraction exhibited the highest specific gravity and the lowest refractive index when compared to the other fractions. The samples labeled extra and first quality possess the strongest and finest odors.

That the chemical composition of the oil may be related to the manner in which the oil is extracted from the flowers has been shown by Glitchitch and Naves (4). They found that when the flowers were initially extracted with petroleum ether and the extract concentrated and distilled, the product was almost free of sesquiterpenes (5). On the other hand, if the flowers were steam distilled, a relatively high concentration of sesquiterpenes was found. This was interpreted by some authors to mean that the sesquiterpenes are formed during distillation from compounds insoluble in petroleum ether and should not be considered true natural products in the strict sense (1).

The chemical composition of ylang-ylang oil was cursorily investigated as early as 1873 working with samples obtained in the Philippines (6–8). In 1932, Glitchitch and Naves (4) using a classical separation identified certain constituents of the extra oil and reported a semiquantita-

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small portions, 10 Gm. (0.04 mole) of I-hydrochloride. The mixture was refluxed for 2 hours, allowed to stand at 26° for 6 hours, decomposed with water and worked up as usual. The oily yellow material (6.5 Gm., 82%) had a strong OH peak in its infrared spectrum. It was benzoylated directly with 20 ml. of benzoyl chloride and 150 ml. of 10% sodium hydroxide solution for 15 minutes, the mixture was decomposed with ice, extracted with dilute hydrochloric acid, the acid solutions were made alkaline and re-extracted with ether. The oily product from the dried ether extract weighed 5 Gm. (50%) and exhibited an ester band in its infrared spectrum. Its hydrobromide crystallized from ethyl formate-isopropyl ether, m.p. 194–197°.

Anal.—Calcd. for $C_{20}H_{23}NO_2 \cdot HBr$: C, 61.54; H, 5.94. Found: C, 61.00; H, 6.10.

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Analysis of the Volatile Components of Ylang-Ylang Oil by Gas Chromatography

By DAVID B. KATAGUE and ERNST R. KIRCH

Five samples of commercially available ylang-ylang oil were analyzed by gas chromatography. Of the various stationary liquid phases used, a 20% Ucon on Chromosorb P(w/w) gave consistently the best separation. The composition of extra, first, second, and third quality fractions and pure oil No. 123 was determined on the basis of relative retention times.

Most essential oils are complex mixtures of individual organic compounds that contain varied functional groups which make complete and detailed analysis relatively difficult or at times impossible using conventional procedures.

Thus, prior to the advent of gas chromatography, the analyses of essential oils were tedious and time consuming. Furthermore, in certain instances in which other methods were used, the analyses were not considered complete (1). The availability and development of gas chromatography as an analytical tool has not only greatly facilitated the separation, and at times the identification, of the constituents of a number of essential oils, but also reduced the time required for analyses (2, 3).

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tive estimate of some of the group components.

A more detailed composition of an absolute ylang-ylang oil was reported by Naves in 1959 (9). This particular oil which was prepared by solvent extraction consisted of the following: *p*-cresylmethylether, 5.7–6.6%; linalool, 6.5–8.1%; methylbenzoate, 6.9–7.4%; benzyl acetate, 19.6–26.5%; and benzyl benzoate, 6.2–9.9%.

Because of the interest in this oil, it was thought advisable to undertake a more detailed analysis of the various commercial samples commonly available in this country. This report deals with the analyses of five samples of ylang-ylang oil by gas chromatography.

EXPERIMENTAL

Apparatus.—The apparatus used in this investigation was the Beckman GC-2 gas chromatograph equipped with a thermal conductivity cell and connected to a Sargent SR recorder with a K-4 disk integrator. The carrier gas was helium. The column used consisted of 6 ft. of copper tubing with a diameter of 1/4 inch.

Column Preparation.—The following stationary liquid phases were used: (*a*) Tide detergent extract (alkyl aryl sulfonates); a commercial sample (20 Gm.) of Tide detergent was extracted in a soxhlet with methanol (200 ml.) and the extract concentrated to a semisolid consistency; (*b*) Apiezon L;[1] diethyleneglycol succinate (DEGS); LAC-R-446 (the adipate polyester of diethyleneglycol partially cross-linked with pentaerythritol); Carbowax 4000;[2] and Ucon 50.[3]

As a solid support, acid-washed Johns-Manville Chromosorb P, 30/60 mesh size, was used. Approximately 20 Gm. of Chromosorb P was required to fill the 6-ft. column employed. In general, a certain weight of a particular liquid phase was dissolved in a minimum amount of an appropriate solvent. To this solution, the proper amount of solid support was added gradually with constant stirring to insure uniform mixing. The mixture was then spread on a large evaporating dish, air-dried at first, and then oven-dried until all the solvent was evaporated. The packing thus prepared was poured slowly into the copper tubing at a uniform rate with persistent tapping to insure uniform packing. Pyrex glass wool was used to plug both ends of the column.

It was determined that 30 ml. of methylene chloride was sufficient to dissolve and deposit 2.0 Gm. of Ucon and 1.0 Gm. of Carbowax on the 20 Gm. of Chromosorb P, while 40 ml. was needed when 2.5 Gm. of Carbowax was used. Thirty milliliters of benzene was used to deposit 1.8 Gm. of DEGS and 1.5 Gm. of LAC-R-446. The same amount of benzene was sufficient to deposit 2.1 Gm. of Apiezon L and 3.0 Gm. of Tide detergent "extract." All solvents employed were of AR grade.

[1] Marketed by James G. Biddle Co., Philadelphia, Pa.

[2] Trade name for polyethylene glycol, marketed by Carbide and Carbon Chemicals Co., a Div. of Union Carbide and Carbon Corp.

[3] Trade name for polyalkylene glycol, marketed by Carbide and Carbon Chemicals Co., a Div. of Union Carbide and Carbon Corp.

TABLE I.—RETENTION TIMES AND RELATIVE RETENTION TIMES OF STANDARD COMPOUNDS ON 20% UCON AT 190°[a]

Compounds[b]	B. p., °C.	Retention Times, Min.	Relative R.T., Min.
α-Pinene	154	2.4	0.296
Furfural	161	4.5	0.555
p-Cresyl methyl ether	176	6.0	0.740
β-Pinene	170	6.8	0.840
Eucalyptol	176	7.0	0.864
Linalool	198	8.1	1.00
p-Cresol	202	9.6	1.18
m-Cresyl acetate	217	9.9	1.22
Linalyl acetate	220	10.0	1.23
Citronellal	208	10.1	1.25
Benzaldehyde	179	10.3	1.27
Methyl benzoate	199	11.0	1.36
Citral	214	12.1	1.49
Bornyl acetate	225	13.4	1.66
Terpineol	220	15.5	1.92
Methyl phenylacetate	220	16.5	2.04
Benzyl alcohol	205	18.0	2.23
Safrole	234	18.8	2.34
Caryophyllene	285	19.0	2.35
Isosafrole	242	19.2	2.37
Phenyl propyl alcohol	219	19.3	2.39
Methyl salicylate	223	19.8	2.44
Ethyl phenyl acetate	226	19.9	2.46
Geraniol	230	20.5	2.54
Benzyl acetate[c]	214	24.0	2.96
Geranyl acetate	248	25.0	3.08
Eugenol methyl ether	249	32.0	3.95
Eugenol	252	34.0	4.20
Isoeugenol	267	38.0	4.81

[a] Flow rate, 40 ml./min.; filament current, 200 ma.; maximum attenuation; linalool, 1.00. [b] Nerol, nerolidol, farnesol, terpenyl acetate, and benzyl benzoate were not eluted even after 70 minutes at the conditions of the experiment. [c] At 756 mm.

Procedure.—The carrier gas flow rate was maintained at 40 ml./min. The temperature ranged from 160 to 190°. The chart speed used was one inch per minute and a filament current of 200 ma. was maintained.

Unless otherwise specified, 0.01 ml. of oil samples were used at maximum sensitivity under the ideal conditions stated above.

For purposes of identification, the comparison of retention times and relative retention times[4] of an unknown with known pure standards were employed. Twenty-nine known compounds were analyzed under conditions identical to that at which the unknown was analyzed. Each peak was then confirmed by the enrichment method. In some cases, infrared spectra were run for confirmation.

The quantitative amount of each peak was measured based on the integrated area under each peak. Computation was based on the integrator. All the percentages as calculated were based on the total eluted.

Samples[5] and Standard Compounds.—The following quality fractions of ylang-ylang oil were analyzed. Their respective refractive indexes at 28° and specific gravities at 24° as determined are listed

[4] The relative retention time is the ratio of the retention time of an unknown constituent to that of a standard constituent.

[5] The authors wish to express their thanks to Fritzsche Brothers Inc., New York, for supplying the five samples of ylang-ylang oil.

Samples	$n_D^{28°}$	Sp. Gr.$^{24°}$
Extra	1.5008	0.963
First quality	1.5041	0.946
Second quality	1.5063	0.936
Third quality	1.5091	0.933
Pure oil No. 123	1.5089	0.915

In addition to the above oils, a number of known pure compounds were analyzed under exactly the same operating conditions as those used for the oils. Table I lists both retention times and relative retention times based on linalool. The corresponding boiling points at 760 mm., unless otherwise stated, are also listed. The compounds are arranged according to their increasing retention and relative retention times. A number of compounds not eluted after 50 to 70 minutes at experimental conditions mentioned are also listed.

RESULTS AND DISCUSSION

Five samples of ylang-ylang oil were analyzed, using various liquid phases in different concentrations with respect to the solid support. The samples analyzed were the extra, first, second, and third quality fraction and pure oil No. 123.

With the extra fraction oil and a 25% w/w Apiezon L on Chromosorb P at 190°, four peaks were obtained, the first two of which only showed good resolution. A change in the column temperature to below or above 190° or a reduction in the amount of Apiezon L to as low as 10% w/w did not improve the resolution. Four peaks at best could be observed at 190° with either a 10% or 25% LAC-R-446, while with DEGS only two broad peaks were obtained. An extract of a detergent (Tide) as the liquid phase was found to decompose at the same temperature and gave incomplete separations.

Carbowax or Ucon gave better resolutions. Both of these liquid phases were investigated in various proportions and at different operating temperatures.

It should be noted that with the extra and first fraction oils at least nine distinct peaks were obtained at 160° and eight peaks at 190° with Carbowax in 10% proportions. Figures 1 and 2 show the typical chromatogram of the extra oil at two temperatures.

Only four, or at best five, peaks (Figs. 3–5) were obtained with the second and third quality fractions and pure oil No. 123, either at 160° or 190°. The results may be due to the absence of the same components as found in the extra and first quality oils, or they may be different components that could not be separated using Carbowax as the liquid phase. It was thought desirable to use another liquid phase.

One of these liquid phases is Ucon (a polyalkylene glycol and its aliphatic diesters). It is reported (10, 11) to be an efficient liquid phase for the separation of components of some volatile oils. With this material in a 20% w/w on Chromosorb P, we were successful in obtaining consistent and better separation of all the oil samples investigated. Figures 6 and 7 represent typical chromatograms obtained with the extra and first quality oils at 190°.

Comparing the results obtained for all the oil samples investigated, it should be noted that the second and third quality as well as the pure oil

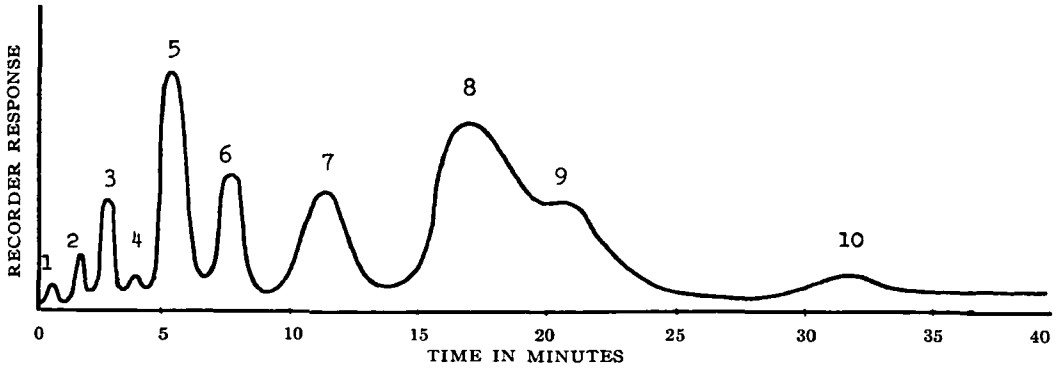

Fig. 1.—Typical chromatogram of extra fraction on 10% Carbowax at 160°.

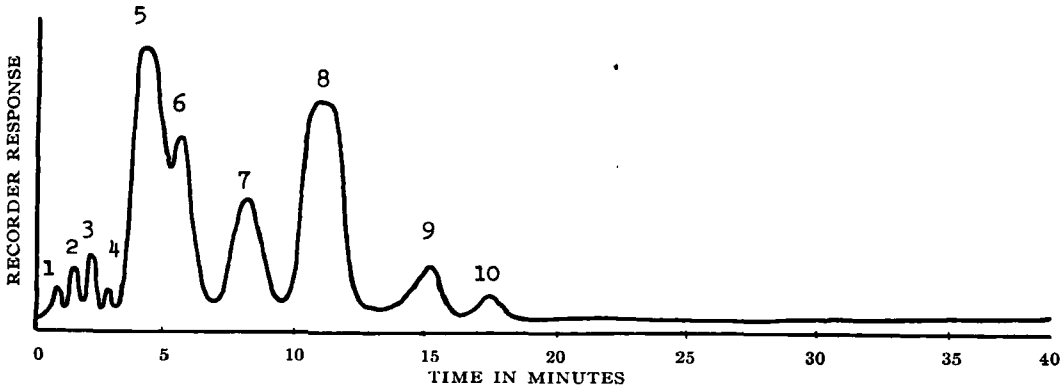

Fig. 2.—Typical chromatogram of extra fraction on 10% Carbowax at 190°.

No. 123 (Figs. 8–10) gave fewer number of peaks. Similar results were obtained at 160° with every one of these oils with the exception that the time required for complete elution was about twice as long.

The primary volatile components of all the oils investigated are listed in Tables II and III giving relative retention times of the peaks and the per cent composition. A particular peak number in a table corresponds to the same number in the various figures. The identity of peaks 2, 4, 5, 6, and 7 was confirmed by the enrichment method using one standard at a time. Peaks 3, 8, and 9 were collected and infrared spectra were run. Peaks 1 and 10, corresponding to low and high boiling components, respectively, were too small

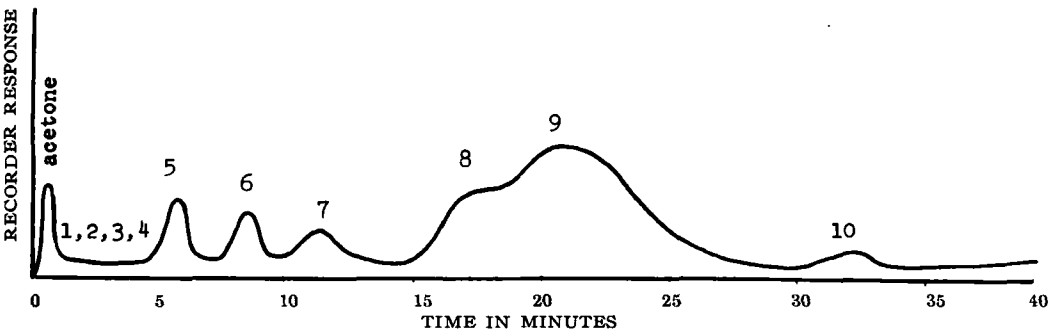

Fig. 3.—Typical chromatogram of second fraction on 10% Carbowax at 160°.

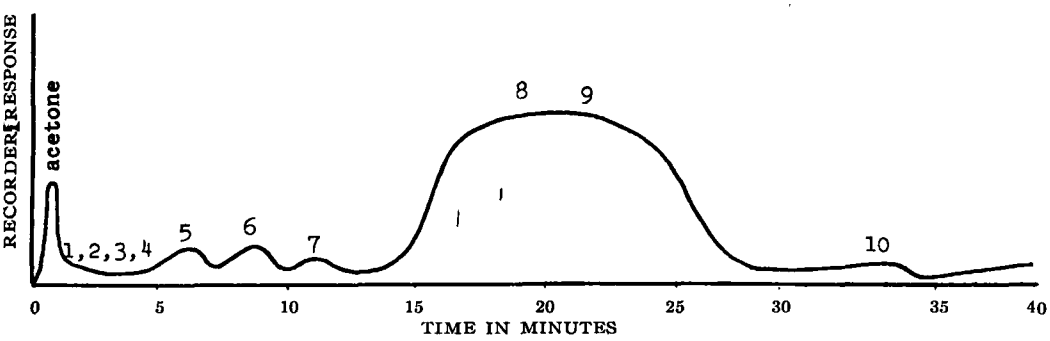

Fig. 4.—Typical chromatogram of third fraction on 10% Carbowax at 160°.

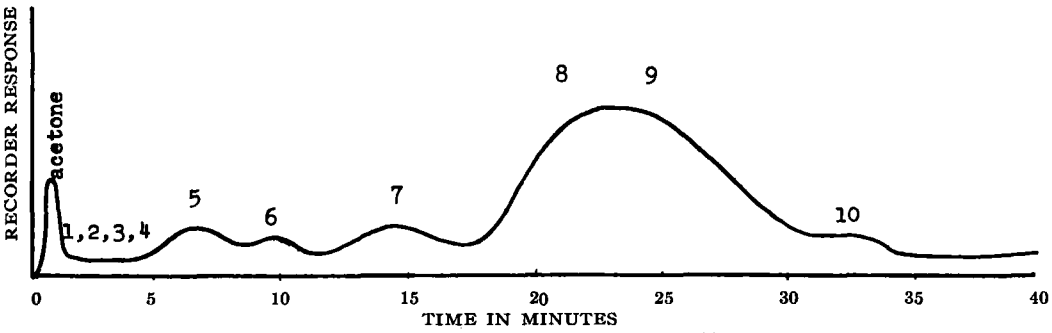

Fig. 5.—Typical chromatogram of pure oil No. 123 on 10% Carbowax at 160°.

TABLE II.—PER CENT COMPOSITION OF YLANG-YLANG OIL, EXTRA AND FIRST FRACTION WITH THEIR RESPECTIVE RELATIVE RETENTION TIMES COMPARED TO STANDARDS[a]

Peak No.	Component	Relative Retention Times Unknown	Relative Retention Times Known	Per Cent Composition Extra	Per Cent Composition First
1	Low boiling components	0.124	...	0.7	0.6
2	α-Pinene	0.296	0.296	1.3	1.2
3	A phenol	0.370	...	1.9	1.6
4	Furfural(?)	0.508	0.555	1.7	2.4
5	*p*-Cresyl methyl ether	0.746	0.740	18.5	13.0
6	Linalool	1.00	1.00	10.8	9.5
7	Methyl benzoate	1.35	1.36	13.2	11.1
8	An aromatic ester	1.82	...	26.7	33.0
9	Geranyl acetate	2.99	3.08	25.1	27.5
10	High boiling components	4.70	...	From 0.5 to 1.0	

[a] Liquid phase Ucon; temperature, 190°; helium flow rate, 40 ml./min.; linalool, 1.00 (8.1 min.).

TABLE III.—PER CENT COMPOSITION OF YLANG-YLANG OIL, SECOND AND THIRD QUALITY FRACTIONS, AND PURE OIL NO. 123 WITH THEIR RELATIVE RETENTION TIMES COMPARED TO STANDARDS[a]

	Peak No. Component	Relative Retention Times Unknown	Relative Retention Times Known	Per Cent Composition Second	Per Cent Composition Third	Per Cent Composition Pure
1	Low boiling components	0.124	...	Traces	No peak	Traces
2	α-Pinene	0.296	...	Traces	No peak	Traces
3	A phenol	0.370	...	Traces	No peak	Traces
4	Furfural(?)	0.508	...	Traces	No peak	Traces
5	*p*-Cresyl methyl ether	0.746	0.740	2.6	0.7	2.4
6	Linalool	1.00	1.00	2.4	0.5	1.9
7	Methyl benzoate	1.35	1.36	2.9	Traces	1.5
8	An aromatic ester	1.82	...	11.8	2.5	7.7
9	Caryophyllene	2.39	2.35	11.5	9.2	12.5
10	Geranyl acetate	2.99	3.08	68.8	86.9	73.9

[a] Liquid phase Ucon; temperature 190°; flow rate, 40 ml./min.; linalool, 1.00 (8.1 min.).

and indistinct for practical confirmation. The retention times for peaks 5, 9, and 10 are within the allowable range as described by James and Bernhard (12, 13).

Alpha-pinene (peak 2) was found to be present both in the extra and first fraction oil. A phenol (peak 3) as indicated by the frequencies of the infrared spectrum as shown in Table IV was also observed in the same two oils. Peak 4 was increased when furfural was added to either one of these oils. *p*-Cresyl methyl ether (peak 5) was present in a higher concentration in the extra as compared to the first fraction, while the amount of linalool (peak 6) was about the same in the two oils. Methyl benzoate as shown by peak 7 is present in slightly higher amounts in the extra fraction.

Terpineol has been reported present in the oil (1). When chromatographed individually under identical conditions used for the oils, a retention time of 15.5 minutes was observed. Peak 8, with a retention time of 14.7 minutes, was not completely enriched. An overlapping, however, was evident. Infrared spectra of peak 8 and standard terpineol were not identical, but indicate an aromatic ester grouping. Infrared spectrum of benzyl acetate compared to peak 8 was similar (Table V).

The constituent represented by peak 9 has a retention time of 24.5 minutes. Two reported components were found to have retention times close to this peak. Geranyl acetate and benzyl acetate were observed to elute after 25 and 24 minutes, respectively. Only geranyl acetate showed

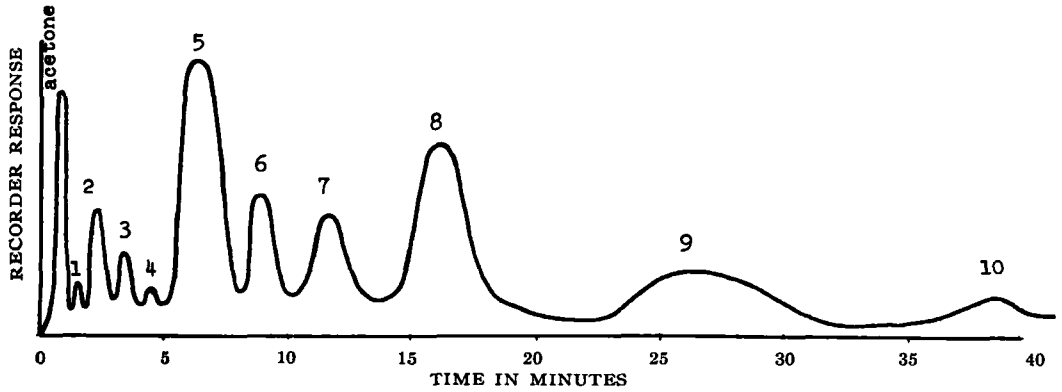

Fig. 6.—Typical chromatogram of extra fraction on 20% Ucon at 190°.

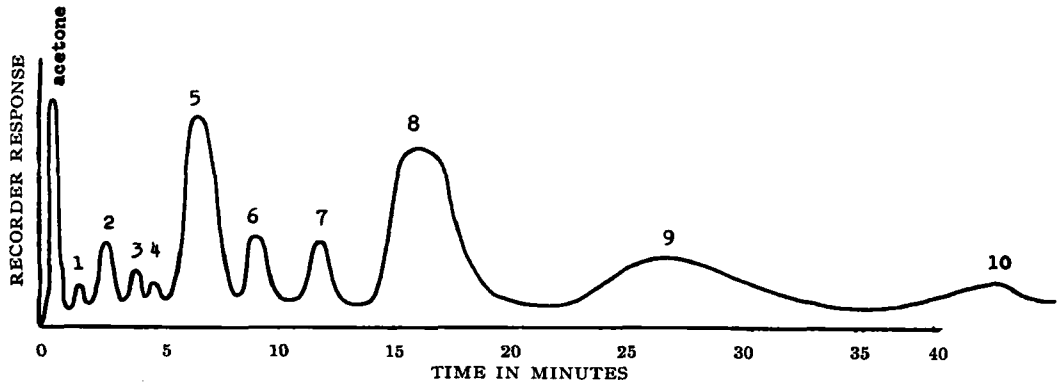

Fig. 7.—Typical chromatogram of first fraction on 20% Ucon at 190°.

an increase in the peak when added to the oil. The infrared spectra of peak 9 and standard geranyl acetate were identical. Table VI shows the infrared frequencies and intensities of peak 9.

Comparing the components of the extra and first fraction oils with the other oils, it should be mentioned that the first four peaks were not observed in the second, third, and pure oil No. 123. The first peak observed (peak 5) was *p*-cresyl methyl ether which was present to the extent of about 2.5% in both the second fraction and pure oil No. 123 samples, but was present only in a concentra-

TABLE IV.—INFRARED FREQUENCIES AND INTENSITIES OF PEAK 3, YLANG-YLANG OIL EXTRA[a]

Frequency, cm.$^{-1}$	Intensity	Frequency, cm.$^{-1}$	Intensity,	Frequency, cm.$^{-1}$	Intensity
3550	Strong	1450	Strong	1100	Strong
3110	Medium	1300	Medium	1030	Strong
1850	Weak	1280	Strong	830	Strong
1630	Weak	1250	Strong	810	Weak
1520	Strong	1180	Medium	750	Strong

[a] A phenol.

TABLE V.—INFRARED FREQUENCIES AND INTENSITIES OF PEAK 8, YLANG-YLANG OIL EXTRA[a]

Frequency, cm.$^{-1}$	Intensity	Frequency, cm.$^{-1}$	Intensity	Frequency, cm.$^{-1}$	Intensity
3100	Medium	1360	Medium	970	Medium
1750	Strong	1250	Strong	930	Weak
1510	Medium	1095	Weak	850	Medium
1450	Medium	1050	Strong	750	Strong
1380	Medium	1000	Medium	700	Strong

[a] An aromatic ester similar to benzyl acetate.

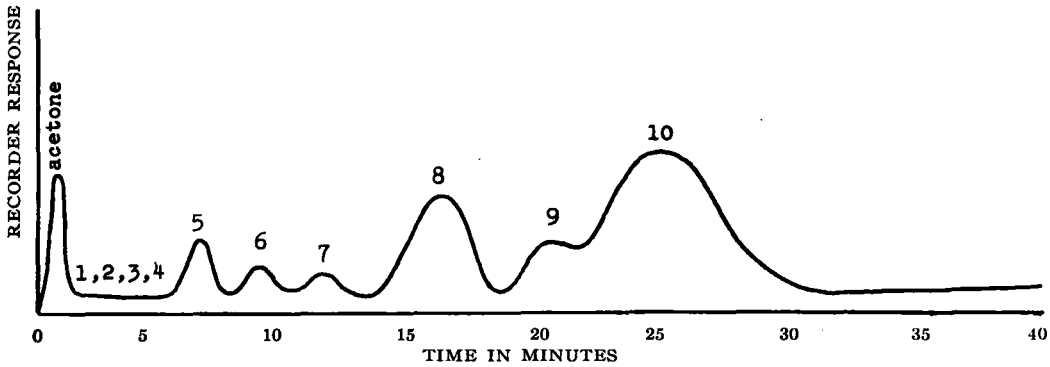

Fig. 8.—Typical chromatogram of second fraction on 20% Ucon at 190°.

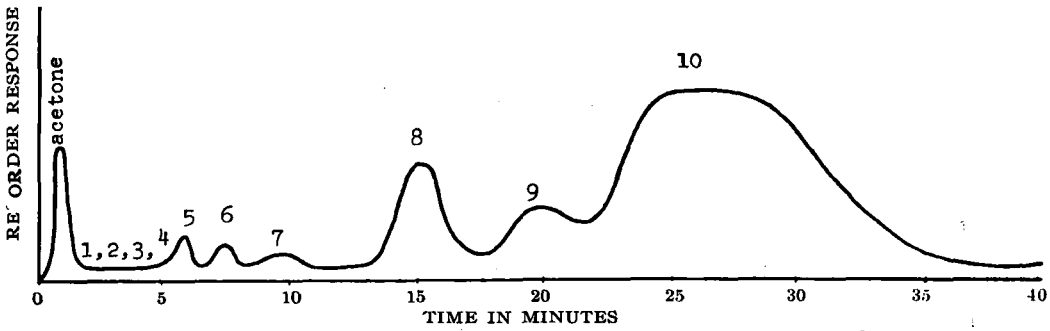

Fig. 9.—Typical chromatogram of third fraction on 20% Ucon at 190°.

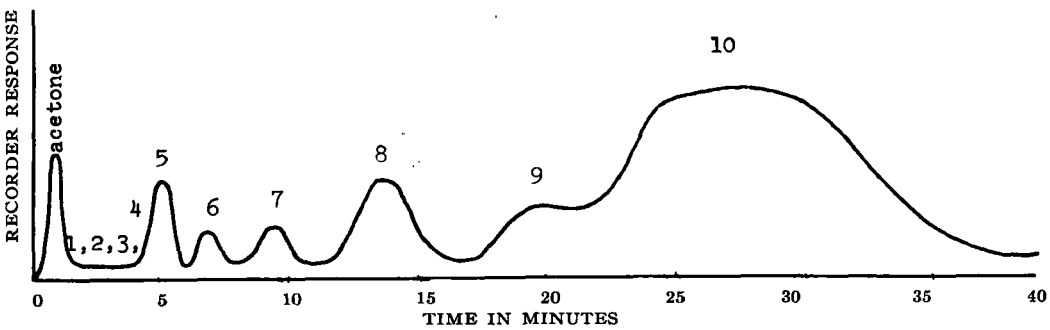

Fig. 10.—Typical chromatogram of pure oil No. 123 on 20% Ucon at 190°.

TABLE VI.—INFRARED FREQUENCIES AND INTENSITIES OF PEAK 9, YLANG-YLANG OIL EXTRA[a]

Frequency, cm.$^{-1}$	Intensity	Frequency, cm.$^{-1}$	Intensity	Frequency, cm.$^{-1}$	Intensity
2950	Strong	1375	Medium	1030	Medium
1720	Strong	1355	Medium	970	Weak
1630	Weak	1310	Weak	890	Weak
1590	Weak	1250	Strong	780	Weak
1450	Medium	1100	Weak	710	Weak

[a] Identical to standard geranyl acetate.

tion of 0.7% in the third fraction. Linalool (peak 6) was slightly higher in concentration in the second fraction than in the pure oil. Again, the amount of this alcohol in the third fraction was almost negligible. Methyl benzoate (peak 7) represents about 2.9% of the second fraction and 1.5% of the pure oil, while only a trace of it was found in the third fraction. An aromatic ester (benzyl acetate), as represented by peak 8, was found to be present in greater amounts in both pure oil No. 123 and the second fraction, as compared to the third fraction.

Caryophyllene has been reported to be one of the sesquiterpenes present in the oil (1). The addition of caryophyllene to the above fraction oils increased peak 9 which eluted at 19.3 minutes. It should be noted that this was not observed in the first or extra oils.

The last peak (peak 10) obtained with second and third quality and pure oil No. 123 represents geranyl acetate and was found to be present in these oils in a relatively high percentage, 68.8 to 86.9%. This amount is about three times as high as was found in the extra or first fraction oils.

It had been mentioned earlier that the literature reports a whole series of compounds present in ylang-ylang oil (1). In practically all cases, the analytical work was carried out on the extra fraction only. The percentages reported by these authors represent several classes of compounds rather than individual compounds. For example, Glitchitch and Naves (4) reported the presence of 0.1 to 0.2% of aldehydes and ketones including benzaldehyde. The latter compound was not found in any of the oils investigated by us. The terpene content reported by the same authors ranged from 0.3 to 0.6%, while we found that the alpha-pinene content of the extra fraction oil as analyzed was in the neighborhood of 1.3%. Some of the samples analyzed in our laboratory showed a concentration of *p*-cresyl methyl ether as high as 18% and a concentration of a phenol as high as 1.9%, while Glitchitch, *et al.*, reported only 3% of phenols and phenol ethers including *p*-cresyl methyl ether. A range of 52 to 64% of alcohols and esters were reported to be present in the extra fraction by the same authors. It was established in this investigation that 10.8% linalool, 13.3% methyl benzoate, 25.1% geranyl acetate, and 26.7% of an aromatic ester (benzyl acetate) were present in the same quality fraction analyzed. Furthermore, Glitchitch, *et al.*, reported about 35% sesquiterpenes to be present in the extra fraction oil. No sesquiterpenes were detected in the extra oil analyzed by us, but caryophyllene was definitely found in the second, third, and pure oil No. 123 in concentration of about 10% (Table III).

SUMMARY

Ylang-ylang oil obtained commercially by distillation of the flowers of *Cananga odorata*, Hook *f et* Thomson, *forma genuina* was analyzed by gas chromatography, using 20% Ucon on Chromosorb P (w/w). The composition of the extra, first, second, and third quality fractions and the pure oil No. 123 was determined on the basis of relative retention times.

The following natural components were identified in ylang-ylang oil: *d*-alpha-pinene, *p*-cresyl methyl ether, linalool, methyl benzoate, geranyl acetate, caryophyllene, and a phenol and an aromatic ester (benzyl acetate). Identification was confirmed by the enrichment method and in certain instances by use of infrared spectra.

On a qualitative basis, the extra and first fractions were similar in that all the components listed above were present except caryophyllene. The second, third, and pure oil 123 oils were similar in that all components listed above were present in significant quantities except *d*-alpha-pinene and a phenol. These two compounds were present in trace amounts in the second and pure oil fractions and were absent in the third fraction.

On a quantitative basis, all five fraction oils analyzed differed in the relative concentration of the components present.

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Some Studies of a Sustained Release Principle

By C. J CAVALLITO, LESTER CHAFETZ, and LOWELL D. MILLER

Urinary excretion of drug after oral dosage of chlorpheniramine as maleate salt and in an oral sustained release formulation was studied, and the urinary excretion and plasma levels of tritium radioactivity obtained after crossover administration of tritium-tagged phenylephrine as hydrochloride and in sustained release formulation were compared. The urinary excretion of both drugs varied widely among individuals, indicating that this parameter is limited in value as a reflection of oral absorption. No relation between blood level and excretion of tritiated phenylephrine was apparent.

THE DEVELOPMENT of oral sustained release forms of medication, which was receiving but scant attention about ten years ago, became a subject of increasing interest following successful introduction of the early products in this field. The increased awareness of the potentials of oral sustained release products has not only led to the development of additional techniques for providing the desired effects, but has also attracted the attention of academic investigators as well as of regulatory agencies. Lazarus and Cooper (1) have provided timely reviews of the subject.

A sustained release product should provide improved performance of a drug as well as the added convenience of fewer doses. Such a product should prevent too rapid a release rate of an absorbable form of drug and permit its gradual availability for absorption over a prolonged period of time. Ideally, these rates of absorption should be tailored as closely as possible to meet the drug requirements of the body without eliciting undesirable effects. There are drugs which obviously have no need for or do not benefit from sustained release formulations.

The interest in our laboratories in oral sustained release products evolved from some simple studies about ten years ago which showed that *Veratrum* alkaloids were relatively well-tolerated orally when utilized as the tannate salt complexes. Investigations showed that a variety of drugs which are organic amines can be converted to stoichiometrically homogeneous pentaamine gallotannates, which are stable, tasteless substances of limited aqueous solubility and which, in the presence of aqueous solutions of electrolytes, provide a gradual release of soluble therapeutic amine salt (2). Since the goal was to provide drug which would be available over a prolonged period of time without initial overdosage, and since stomach acid could lead to too rapid a release of amine from its tannate, it was determined that intimate combination with a polymeric, polyanionic agent, such as polygalacturonic acid, could protect the tannate from too rapid solution in acids. Using as a guide the responses in humans administered a variety of dosage preparations, there was developed an oral sustained release principle which utilizes amine tannates and polygalacturonic acid in such proportions that rate of release and absorption of the amine drug can occur gradually over a wide range of pH values.[1] The polygalacturonic acid, which prevents too rapid a solubilization of amine from the tannate in the acid media, itself becomes more soluble as the gut becomes less acid. Ultimately, all components may become individually dissolved. Since the specially prepared solid and liquid dosage forms disperse to a colloidal state in aqueous media, they should tend to empty from the stomach gradually and continue to be available at intestinal sites of absorption over an extended period of time. [Wagner (3) has critically reviewed the effect of particle number and size as related to gastric emptying and the onset of therapeutic effect of sustained release drugs.]

A variety of therapeutic and chemical categories of amine drugs have been converted to this type of sustained release product and evaluated largely on the basis of their clinical performance relative to that of the usual, nonsustained release forms of the drugs (4–16). Following demonstrated performance in man, ancillary studies were carried out with these preparations in an attempt to obtain more quantitative measures of drug action. Some of these *in vivo* studies are reported and their uses and limitations discussed.

EXPERIMENTAL

Urinary Excretion of Chlorpheniramine.—Urinary excretion of chlorpheniramine was measured after the administration of the drug in nonsustained re-

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[1] This type of sustained release product is referred to by the trade name of Durabond by Irwin, Neisler & Co.

lease and in sustained release forms. The sustained release form utilized the tannate salt[2] of chlorpheniramine, and chlorpheniramine maleate served as the more rapidly absorbable form of the drug. Each subject was given, at weekly intervals, unit doses of 10.2 mg. of chlorpheniramine maleate (7.17 mg. base) in small gelatin capsules and 30 mg. of chlorpheniramine tannate (13.4 mg. base). Following ingestion of drug, urine samples were voided at specified intervals corresponding to times at which urine blanks were obtained on each subject. Urine samples were made alkaline with sodium hydroxide, and the drug was extracted into chloroform. The chloroform was washed with pH 10 borate buffer to reduce the blank readings, then extracted with dilute aqueous acid. The concentration of chlorpheniramine was estimated from the absorbance of the acid solution at 265 mμ determined with a Beckman model DU spectrophotometer. The isolation procedure gave satisfactory recoveries (98.4%) of chlorpheniramine four hours after its addition to urine.

Plasma Levels and Urinary Excretion Rate of Tritium-Labeled Phenylephrine.—Several years ago, a study in humans was initiated for the purpose of determining the rates of appearance of a radioactive drug component in blood following its oral administration in conventional and in Durabond-type preparations. Phenylephrine with random tritium tagging was used as the test drug. The investigation was conducted in cooperation with the Nuclear Science and Engineering Corporation, and the technique used in the pilot study was described by Bogner and Walsh (17). A more extensive study was completed more recently in which both blood levels and urinary excretion of drug were measured.

Twelve adult male subjects with no known disorders of absorption or excretion were used in the study. They were given doses of tritiated phenylephrine as hydrochloride and as tannate in similar appearing tablets at intervals of a week. Six subjects (age range: 23–39 years; weight range: 150–206 lbs.) were given doses of phenylephrine tannate equivalent to 15.68 mg. base, and phenylephrine hydrochloride corresponding to 7.4 mg. base was selected for comparison. These were doses which in practice provided comparable initial therapeutic responses and which in earlier pilot studies yielded initially comparable drug blood levels.[3] In another six subjects administered half these doses, the lower radioactivity levels showed relatively large standard deviations and were of questionable quantitative significance. Even with the higher doses, the standard deviations were appreciable.

All doses were given after an overnight fast, and the subject was allowed four ounces of water immediately after taking each tablet. Identical light meals were ingested by each subject 1 hour after dose administration. Blood samples (15 ml.) were obtained at indicated intervals from the antecubital veins. Plasma was immediately separated by centrifugation and frozen until analyzed. Total urine collections were made as voided at five interval periods up to 48 hours. Details of the analytical procedures used for determination of tritium radioactivity in plasma and urine samples are described elsewhere (17).

RESULTS AND DISCUSSION

Chlorpheniramine Urinary Excretion.—The quantities of chlorpheniramine found in the urine of four subjects after oral dosage of chlorpheniramine maleate and of three of the same four (one subject became unavailable) after oral dosage of the drug in the tannate form are shown in Table I.

Table I.—Interval Excretions of Chlorpheniramine[a] in Urine After Oral Dose

Hours	Subject I	Subject II	Subject III	Subject IV
	Chlorpheniramine Maleate[b]			
2	0.00	0.15	0.02	0.05
4	0.39	0.28	0.07	0.00
6	0.84	0.52	0.12	0.10
8	0.64	0.44	0.07	0.05
10	0.62	0.40	0.07	0.06
12	0.60	0.25	0.06	0.28
15	0.30	0.25	...	0.07
24	0.70	0.54	0.43	0.29
28	0.90	0.20	...	...
34	1.00	0.06	...	...
	Chlorpheniramine Tannate[c]			
3	0.56	0.30	0.00	...
6	0.41	0.76	0.40	...
9	0.19	0.33	0.59	...
12	0.66	0.72	0.46	...
24	1.79	1.43	0.90	...
29	0.77	...	...	...
32	...	0.48	0.63	...

[a] As mg. base. [b] 7.17 mg. base. [c] 13.4 mg. base.

The doses of each form selected were those which represented equivalents in terms of initially induced therapeutic response. The data for the rapidly absorbable form show a considerable difference and irregularity among subjects in their period of maximum excretion and in the total amount of drug excreted. These differences appear to be less after dosage of the sustained release form of the drug.

Variations in Urinary Excretions.—The wide variation among individual excretions of chlorpheniramine with these few subjects raised questions as to the significance of such measurements as reflections of rate of absorption of this antihistaminic and possibly of other drugs. A review of the published literature purporting to relate urinary excretion to drug absorption rates showed that wide individual variations also were evident from the data of others but that usually little if any note was made of this in plotting cumulative averaged values. Following oral administration of a drug, wide variations are evident among individual excretions, particularly during the first few hours, and as cumulative values are shown with time, the interval excretion variations tend to cancel one another to provide more uniform total recoveries. Wide variations in excretion (of drug or metabolite) following oral administration of single doses of

[2] Reference to tannates throughout this report denotes their incorporation with polygalacturonic acid in Durabond formulations.

[3] From a practical use basis, we were interested in the relative duration of action of dosage forms producing an adequate therapeutic response without undesirable side reactions.

drugs, particularly during the earlier intervals, may be illustrated by published data (Table IV) (18–26). All of these reports (except reference 26) measured only urinary excretion of drug or metabolites and not drug blood concentrations. A more comprehensive study utilizing sulfaethylthiadiazole was reported by Swintosky, *et al.* (26–28), in which both blood and urine drug levels were measured. This drug, which is virtually all excreted in 72 hours, also showed considerable individual variations in excretion levels in the first 3-hour period even with parenteral administration of the drug and these variations tended to decrease with increase in administered dosage.

Phenylephrine Plasma Levels and Urinary Excretion Rates.—The tritiation tagging technique was used to measure blood concentrations and urinary excretions of drug following administration of soluble and tannate forms of phenylephrine in that there was not available a sufficiently sensitive chemical method at the time of the study. [Since the completion of this study a fluorometric method for the determination of phenylephrine in blood has been reported (29).] Although there is no absolute evidence that the tritium level represents unchanged phenylephrine, nevertheless the measure of tritium radioactivity does directly reflect phenylephrine which has been absorbed and does permit comparison of the dosage forms. The tritium-labeled phenylephrine had been recrystallized under conditions which removed labile tritium (17) and minimized the possible influence of tritium exchange during the experimental period. The tritium activity values, expressed in terms of micrograms of phenylephrine base per 100 ml. of plasma, are given in Table II for phenylephrine hydrochloride and for the tannate. The mean values are graphically represented in Fig. 1. Trit-

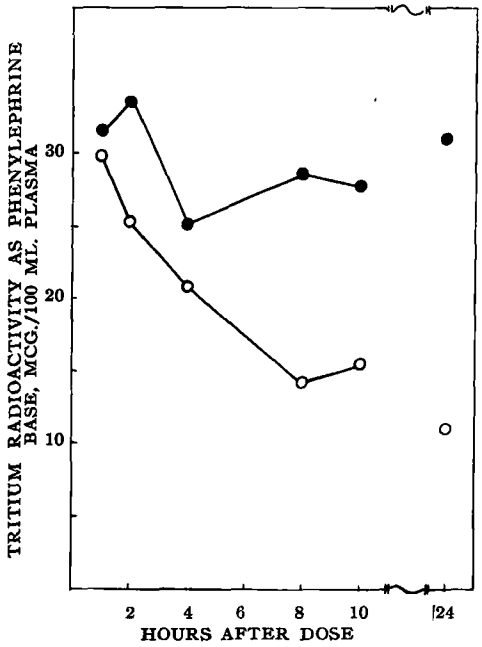

Fig. 1.—Mean plasma levels of tritium radioactivity expressed as phenylephrine base after oral dose of 7.38 mg. base as hydrochloride, O, and 15.68 mg. base as tannate, ●.

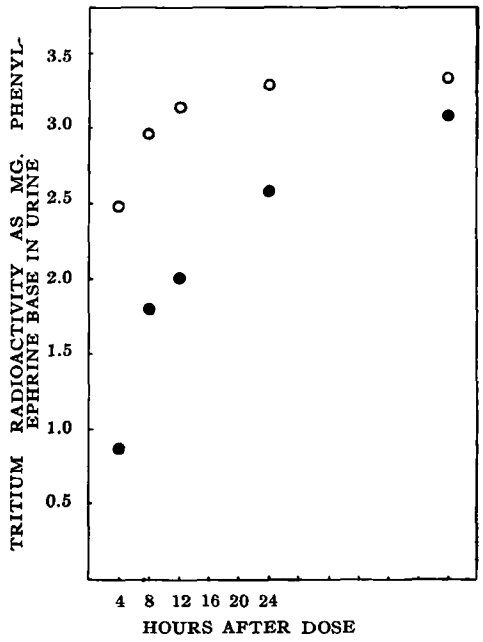

Fig. 2.—Cumulative mean tritium radioactivity expressed as phenylephrine base after oral dose of 7.38 mg. base as hydrochloride, O, and 15.68 mg. as tannate, ●.

TABLE II.—TRITIUM ACTIVITY IN PLASMA AFTER DOSE OF TAGGED PHENYLEPHRINE[a]

Hours	Subject I	II	III	IV	V	VI	Mean ± S.D.
			Phenylephrine Hydrochloride[b]				
1	36.4	47.3	29.2	24.1	16.8	25.5	29.8 ± 10.7
2	31.3	29.9	31.3	19.0	23.3	16.8	25.3 ± 6.5
4	26.2	17.5	30.6	16.8	17.5	16.8	20.8 ± 6.0
8	7.3	18.2	16.8	10.2	17.5	15.3	14.2 ± 4.5
10	10.9	18.2	29.9	14.5	9.4	10.2	15.5 ± 7.7
24	18.2	5.8	13.9	5.8	10.9	11.7	11.0 ± 4.8
			Phenylephrine Tannate[c]				
1	29.9	29.9	34.3	39.8	36.2	39.8	31.6 ± 9.4
2	36.2	34.3	37.9	25.2	18.0	50.5	33.6 ± 11.6
4	10.8	25.2	32.6	23.5	32.6	27.1	25.2 ± 8.0
8	23.5	30.8	32.6	18.0	43.4	23.5	28.6 ± 8.9
10	25.2	23.5	39.8	25.2	19.9	32.6	27.8 ± 7.2
24	34.3	45.1	23.5	25.2	25.2	32.6	31.0 ± 8.1

[a] Expressed as mcg./100 ml. plasma. [b] 7.38 mg. base. [c] 15.68 mg. base.

ium levels expressed as phenylephrine base recovered in urine during intervals following ingestion of the hydrochloride and tannate forms are presented in Table III for these subjects, and the mean cumulative amount of radioactivity expressed as phenylephrine base is plotted for both dosage forms in Fig. 2. The plotting of mean cumulative values is a rather conventional procedure but, as noted earlier, this can leave a misleading impression of uniformity or consistency of excretion patterns.

Fairly similar mean tritium blood levels are provided (Fig. 1) at the first hour by the doses utilized of rapidly soluble and sustained release forms of phenylephrine. The blood levels are definitely maintained closer to this initial value for a considerably greater time from the sustained release than from the rapidly soluble drug form. Drug blood concentrations may not necessarily be directly related to therapeutic responses but the results are at least consistent with the clinical reports on the sustained effects of the principle.

If one compares tritium interval excretion with average blood levels during that interval (the shorter the interval the more valid the comparison, however, physical limitations should be recognized), there is observed a rather poor correlation. Particularly puzzling is the much greater difference in urinary excretion than in the blood levels from the two drug forms in the first 4 hours. Thus, although the two drug forms show no great dif-

TABLE III.—TRITIUM RADIOACTIVITY IN URINE AFTER DOSAGE OF TAGGED PHENYLEPHRINE[a]

Hours	Subject I	II	III	IV	V	VI	Mean ± S.D.
				Phenylephrine Hydrochloride[b]			
0–4	3.66	3.02	1.42	2.36	3.36	1.08	2.48 ± 1.05
4–8	0.40	0.23	0.17	0.24	0.75	1.07	0.48 ± 0.36
8–12	0.10	0.07	0.19	0.21	0.15	0.30	0.17 ± 0.08
12–24	0.21	0.31	0.44	0.28	0.44	0.39	0.35 ± 0.09
24–48	0.11	0.09	0.12	0.12	0.22	0.24	0.15 ± 0.14
				Phenylephrine Tannate[c]			
0–4	2.46	0.74	0.78	0.29	0.25	0.71	0.87 ± 0.81
4–8	0.24	0.92	0.85	0.58	1.85	1.08	0.92 ± 0.54
8–12	0.16	0.17	0.13	0.28	0.28	0.22	0.21 ± 0.06
12–24	0.26	0.89	0.46	0.35	0.91	0.59	0.58 ± 0.27
24–48	0.20	0.70	0.60	0.50	0.38	0.61	0.50 ± 0.18

[a] Expressed as mg. phenylephrine. [b] 7.38 mg. base. [c] 15.68 mg. base.

TABLE IV.—SOME REPORTED RANGES IN DRUG EXCRETIONS WITH EMPHASIS ON EARLY INTERVALS

Reference	Drug	Oral Dose, mg.	Number Subjects	Period, hr.	Excretion Range Min.	Max.
18	Aspirin B	600	14	1	6.1	34.0
18	Aspirin D	600	14	1	1.5	28.3
18	Aspirin E	600	14	1	4.2	17.1
18	Aspirin B	600	14	9	121	388
18	Aspirin D	600	14	9	146	362
18	Aspirin E	600	14	9	178	335
19	Aspirin	1000	9	1	7.3	28.2
19	Al aspirin	1120	9	1	3.6	8.4
19	Aspirin	1000	5	1	18.9	39.5
19	Aspirin	500	5	1	8.24	20.6
19	Al aspirin	1120	5	1	1.34	13.6
20	Aspirin	650	5	1	20.6	45.6
20	Aspirin	650	5	4	189.0	251.2
20	Benzylpenicillin	100,000[a]	5	0.5	0	3100[a]
20	Benzylpenicillin	100,000[a]	5	5	10,200[a]	26,300[a]
20	Sulfaethylthiadiazole	1000	7	0.5	0	42.5
20	Sulfaethylthiadiazole	1000	7	8	305.7	589.1
21	Phenylpropanolamine hydrochloride	50	3	2	7.4	16.6
21	Phenylpropanolamine hydrochloride	50	3	24	45.2	50.5
22	Tolbutamide	250	4	1	1	11
22	Tolbutamide	250	4	3	4	19
22	Tolbutamide (Na)	250	4	1	9	28
22	Tolbutamide (Na)	250	4	3	81	123
23	Na paraaminosalicylate (solution)	500	7	2	161	306
23	Na paraaminosalicylate (solution)	500	7	4	261	375
24	Tetracycline hydrochloride	200	6	1	1.8	5.5
24	Tetracycline hydrochloride	200	6	2	6.5	20.8
24	Tetracycline hydrochloride	200	6	4	17.5	36.0
24	Tetracycline·mucic acid	200	6	1	0	2.7
24	Tetracycline·mucic acid	200	6	4	2.2	23.0
25	Nicotinic alcohol ("Timespan")	300	3	4	3	3.6
25	Nicotinic alcohol ("Timespan")	300	3	24	27	34.2
26	Sulfaethylthiadiazole	500[b]	4	3	29	113
26	Sulfaethylthiadiazole	1000[b]	4	3	204	296

[a] Units. [b] Intravenous.

ference in mean blood levels during the first 4 hours, the mean urinary excretion values are in the ratio of 2.48 to 0.87 (hydrochloride to tannate) for this interval. From 4–48 hours the mean urinary excretion ratio was 1.15 to 2.21 (hydrochloride to tannate) and this more closely reflected the blood level patterns.

The question arose as to whether a peak blood level might have been provided by the rapidly soluble drug form in less than 1 hour and that this led to the large excretion. To determine this, several of the same subjects were again given the hydrochloride form of the drug about 3 months later, and blood levels were measured after one-half hour as well as 1 hour. In four of five repeated subjects the blood levels were considerably lower in the half-hour reading than after 1 hour; in subject III blood levels were negligible at both periods; whereas in the previous test the same subject gave a 1-hour level near the mean for the group. The higher urinary excretion in the comparative study would thus be difficult to explain on a basis of very high blood levels within a half hour or less after drug administration.

The new data reported here and most of that previously published by others show that urinary excretion of drugs and their metabolites varies very widely among individuals, particularly with lower dosage of drugs and during the first few hours after administration. In those few instances in which both drug blood concentrations and urinary excretions are measured, there is found to be rather inconsistent correlation among individuals. The use of drug excretion rate measurements as reflections of oral absorption from sustained release formulations is of questionable value.

SUMMARY AND CONCLUSIONS

With the drugs, chlorpheniramine and phenylephrine, urinary excretion in man shows wide individual variations and is indicated to be an unreliable reflection of rate of oral absorption. This also is evident from published data with other drugs, particularly as pertains to the first few hours after drug administration. Plotting of cumulative excretion values tends to obscure interval variations.

Rapidly absorbable and sustained release formulations containing tritiated phenylephrine were administered in doses which provided similar early blood levels. There is little relation between urinary excretion and blood concentrations of tritiated drug during the first few hours. The sustained release form maintained blood levels at near the first hour's value for a considerably longer period of time than did the rapidly soluble drug form.

More information is needed with regard to interrelationships of absorption, blood concentrations, and urinary excretion of rapidly soluble forms of drugs before one can assume that urinary excretion profiles are true reflections of rates of absorption of drugs from oral sustained release preparations. The wide variations among these parameters in the first few hours after drug administration particularly merits more attention.

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Adsorption of Sorbic Acid by Plastic Cellulose Acetates

By WITOLD SASKI

The adsorption of sorbic acid from aqueous solutions by plastic cellulose acetate and cellulose triacetate was studied. Adsorption isotherms at 25 ± 0.2° were determined and Langmuir constants were calculated. The method presented is recommended as satisfactory for use in comparative studies of adsorption of drugs, fungistatic and bacteriostatic agents by plastic materials. Adsorption of sorbic acid as a function of pH was studied and was shown to decline to zero in the vicinity of the point of maximum ionization of sorbic acid.

PLASTICS have been rapidly replacing the conventional materials for the manufacture of containers and various devices used in pharmaceutical and medical practice. The common belief that plastics are inert and, therefore, safe for any use is unjustified. It has been demonstrated that they may cause a tissue sensitivity or toxic response when used as surgical implants. A constituent may be released from the plastic to the solution which is to be injected into the patient when plastic devices such as syringes and tubings are used. A plastic device or container may bind or adsorb a significant quantity of the drug, bacteriostatic or fungistatic agent present in the formulation and affect the potency and perhaps even the stability of the product. A review of the literature on the subject was published by Autian (1).

Plastics, by definition (2), are a large and varied group of materials which consist of, or contain as an essential ingredient, a substance of high molecular weight which while solid in the finished state, at some stage in its manufacture is soft enough to be formed into various shapes, usually through the application, either singly or together, of heat and pressure. The thermosets are plastics which harden and form a permanent shape upon heating. On reheating and cooling, these thermosets will no longer have the same characteristics they originally had. In contrast to the thermosets, the thermoplastics can be reheated and cooled a number of times without materially altering their original characteristics. Fluorocarbons, polyethylenes, vinyls, nylons, styrenes, acrylics, and cellulosics are examples of thermoplastics.

To attain the desirable properties of plastic materials, various additives are needed. They may consist of plasticizers, fillers, and stabilizers. In cellulose plastics (3), the plasticizers most frequently used are the phthalates—diethyl, dibutyl, dioctyl, and methoxyethyl phthalate. Although triphenyl phosphate is a relatively poor plasticizer when compared with the phthalates, it is frequently used in order to produce a more rigid sheet or to reduce the flammability of the product. The proportions of the above plasticizers used may vary widely, frequently running from as low as 8 to as high as 40 parts per 100 parts of cellulose ester, depending upon the physical properties desired in the finished sheet. In addition to plasticizers, coloring agents, ultraviolet light absorbers, and various types of surface coatings to improve slip characteristics may also be employed (4).

The present study set out to determine the extent of possible adsorption of the fungistatic agent, 2,4-hexadienoic (sorbic) acid, by plastic cellulose acetates, and to develop a method suitable for comparative evaluation of the adsorptive capacities of different plastic materials.

EXPERIMENTAL

Apparatus and Materials.—The following apparatus was used: Beckman DU spectrophotometer, Photovolt model 110 electronic pH meter, Christian Becker analytical balance, constant temperature water bath, and the Burrell wrist-action model DD shaker. The following materials were used: plastic cellulose acetate sheets, 0.01 in. thick;[1] plastic cellulose triacetate sheets, 0.01 in. thick;[2] sorbic acid;[3] alcohol U.S.P.; 1 *N* hydrochloric acid, and 1 *N* sodium hydroxide.

Methods of Analysis.—Sorbic acid was recrystallized and the melting point determined according to standard procedures. The quantitative determinations of sorbic acid in solutions were performed using standard spectrophotometric techniques. A 256 mμ wavelength of maximum absorption was used (5). In all instances, the solutions to be assayed were adjusted to contain 3 mcg. or less of sorbic acid per ml.

Adsorption as a Function of Time.—Strips of

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[1] Marketed as Kodacel A29, by Eastman Kodak Co.
[2] Marketed as Kodapak IV, Type F 401, by Eastman Kodak Co.
[3] Marketed as Sorbistat by Chas. Pfizer & Co., Inc.

plastic cellulose acetate sheet, 0.01 in. thick and approximately 1 in. × 7 in. in size, were weighed on the analytical balance and placed into glass-stoppered cylinders containing exactly 100 ml. of a 0.150% (w/v) sorbic acid solution in distilled water. Seven cylinders were used: six with plastic strips, and one as a control and to check on possible sorbic acid degradation. The cylinders were placed in the constant temperature bath adjusted to 25 ± 0.2° and subjected to constant and uniform shaking by means of the Burrell wrist-action model DD shaker set in position 1.

The procedure developed in this investigation was as follows. One-milliliter portions of the solution were withdrawn from each cylinder and diluted to 500 ml. in a volumetric flask to obtain the desired sorbic acid concentration. The adsorbance of the samples was then determined, using quartz 1-cm. cells in the Beckman DU spectrophotometer. The results are shown in Table I and Fig. 1. It became apparent that the equilibrium concentration of sorbic acid in aqueous solution in the presence of plastic cellulose acetate is reached within 14 days. This finding has been utilized in the subsequent experiments.

Adsorption as a Function of Concentration.— Following the procedure described, strips of cellulose acetate were placed into cylinders containing 100 ml. of aqueous solutions of sorbic acid. Triplicate samples of each of five concentrations, 0.050, 0.075, 0.100, 0.125, and 0.150% (w/v), were used. This experiment was carried out as before in the constant temperature water bath at 25 ± 0.2° and with the shaker set at the same speed. Since the previous experiment determined that the equilibrium concentration of sorbic acid was reached within 14 days, no samples were tested until this time had elapsed. Following the identical procedure, strips of plastic cellulose triacetate were subjected to the same treatment. The results are reported in Table II. Figure 2 shows the adsorption iso-

TABLE I.—ADSORPTION OF SORBIC ACID BY PLASTIC CELLULOSE ACETATE AS A FUNCTION OF TIME[a]

No. Days Elapsed	Mg. Acid per Gm. Plastic
3	16.00
5	26.15
7	32.70
9	36.34
10	38.00
12	39.50
14	40.00
16	40.00
20	39.60

[a] Concentration of the aqueous solution: 0.150% (w/v); temperature: 25 ± 0.2°.

TABLE II.—ADSORPTION OF SORBIC ACID AS A FUNCTION OF CONCENTRATION[a, b]

	Acid Concn., %	x/m	c	$\frac{c}{x/m}$
Cellulose acetate	0.050	16.05	26.50	1.65
	0.075	27.50	52.25	1.90
	0.100	30.34	64.54	2.15
	0.125	38.00	87.40	2.30
	0.150	39.51	95.63	2.45
Cellulose triacetate	0.050	9.56	37.76	3.95
	0.075	11.30	60.00	5.31
	0.100	13.20	85.50	6.47
	0.125	14.16	104.16	7.35
	0.150	15.34	129.30	8.43

[a] Temperature 25 ± 0.2°. [b] x/m = mg. of sorbic acid adsorbed per Gm. of plastic; c = equilibrium concentration in mg. sorbic acid per 100 ml.

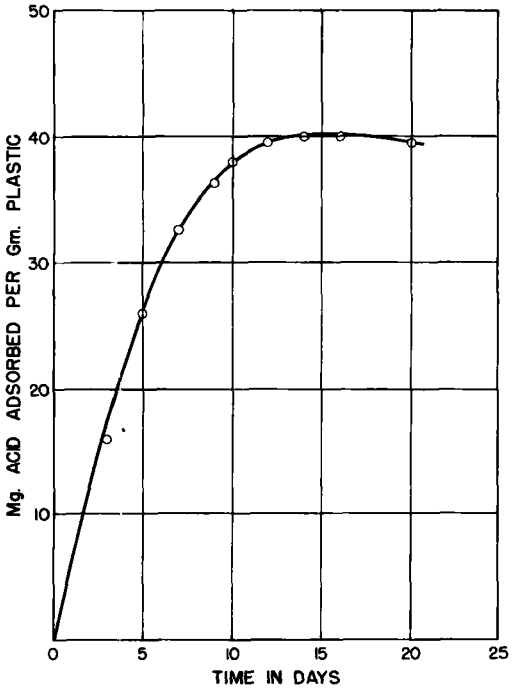


Fig. 1.—Adsorption of sorbic acid by plastic cellulose acetate as a function of time.

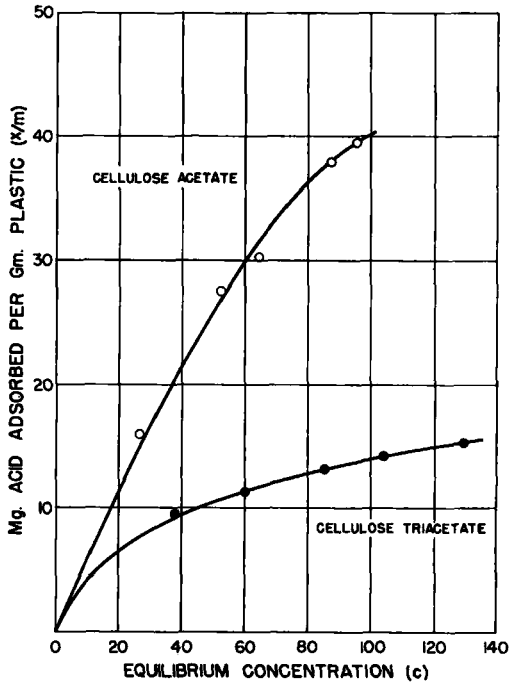


Fig. 2.—Langmuir adsorption isotherms for plastic cellulose acetate and cellulose triacetate.

therms for both plastic materials. Figure 3 shows the linear form of the Langmuir adsorption isotherms for both plastics.

Adsorption as a Function of pH.—Following essentially the same procedure, strips of plastic cellulose acetate were placed into glass cylinders containing 100 ml. of a 0.150% (w/v) sorbic acid aqueous solution adjusted to various pH levels by the addition of 1 *N* solution of hydrochloric acid or 1 *N* solution of sodium hydroxide, all in triplicate. The solutions were not buffered, in order to avoid the possibility of interference with adsorption by the buffer salts. The pH of each solution was determined with the Photovolt model 110 electronic pH meter, both at the outset of the experiment and at the end of the apparent equilibration period of 14 days. The results are reported in Table III and graphically presented in Fig. 4, which represents the pH data obtained after contact with the plastic. Sorbic acid percentage ionization values were calculated and shown in Table III.

Adsorption as a Function of Solvent System.—The effect of ethyl alcohol concentration on sorbic acid adsorption by plastic cellulose acetate was determined. The concentration of sorbic acid in all samples was 0.150% (w/v) and the concentrations of the alcohol were 0, 9.5, 19.0, 38.0, and 76.0 (v/v), respectively, with triplicate samples of each concentration being tested, together with an appropriate number of controls. The procedure followed was that previously described. The results are graphically presented in Fig. 5.

RESULTS AND DISCUSSION

After having determined the time necessary to attain equilibrium, the effect of sorbic acid concentration on adsorption by plastic cellulose acetate and cellulose triacetate was studied. This is presented in two ways. First, by plotting the equilibrium concentration of sorbic acid in mg. per 100 ml. of solution (c) as abscissa against the number of mg. of sorbic acid adsorbed by one Gm. of plastic, as shown in Fig. 2.

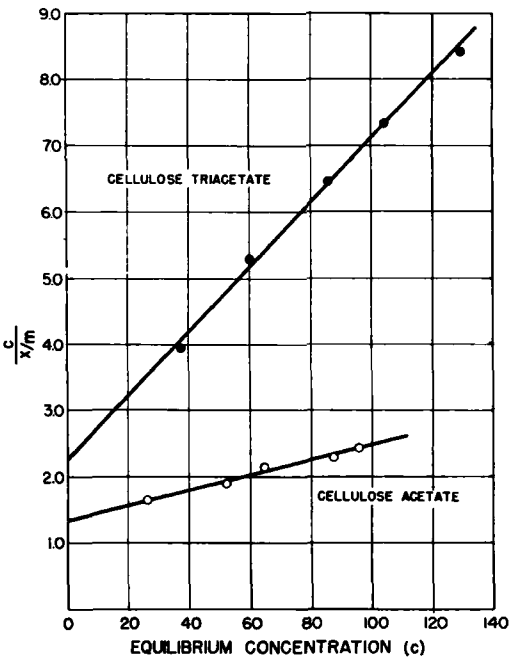


Fig. 3.—Linear form of the Langmuir adsorption isotherms for plastic cellulose acetate and cellulose triacetate.

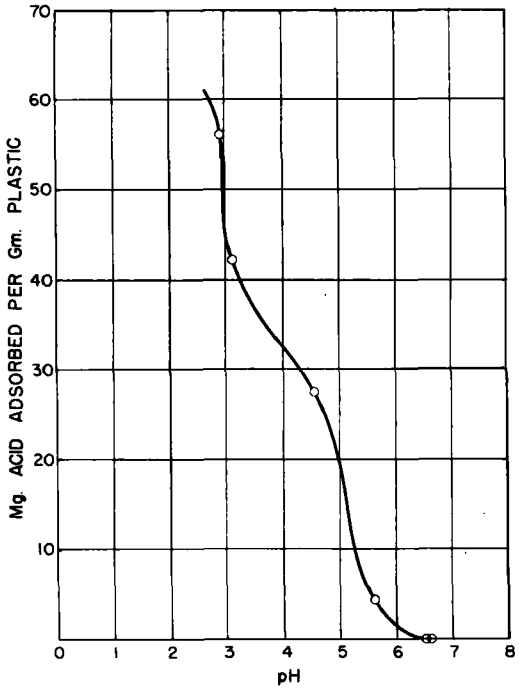


Fig. 4.—Adsorption of sorbic acid by plastic cellulose acetate at various pH levels.

TABLE III.—ADSORPTION OF SORBIC ACID BY PLASTIC CELLULOSE ACETATE AT VARIOUS pH LEVELS[a]

Av. Wt. of Plastic in Gm.	pH: Before Contact with Plastic	pH: After Contact with Plastic	Calculated Percentage Ionization[b]	Sorbic Acid: Mg. Adsorbed	Sorbic Acid: Mg. Adsorbed per Gm. Plastic
1.1140	2.60	2.90	1.36	62.5	56.10
1.1854	3.30	3.10	2.14	50.0	42.18
1.1763	4.50	4.55	38.16	32.5	27.63
1.1294	5.52	5.60	87.25	5.1	4.51
1.2755	7.30	6.50	98.21	0	0
1.2095	10.80	6.60	98.57	0	0

[a] From a 0.150% (w/v) solution. Temperature 25 ± 0.2°. [b] Calculated from the equation (6):

$$\text{Percentage ionization} = \frac{100}{1 + \text{Antilog (pKa} - \text{pH)}}$$; pKa of sorbic acid is 4.76.

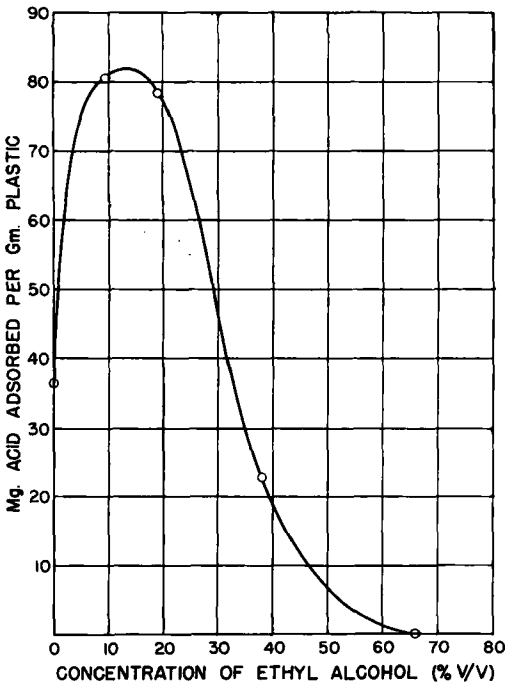


Fig. 5.—Effect of ethyl alcohol on the adsorption of sorbic acid (0.150% w/v) by plastic cellulose acetate.

The second method of presenting these data is by plotting the equilibrium concentration against the ratio of the equilibrium concentration to the number of mg. of sorbic acid adsorbed by one Gm. of plastic, as shown in Fig. 3. This type of graph, which represents the linear form of the Langmuir equation (7), is particularly useful. Derived from the equation

$$\frac{x}{m} = \frac{abc}{1 + a\,c}$$

where x/m = mg. of sorbic acid adsorbed per Gm. of plastic, c = equilibrium concentration in mg. sorbic acid per 100 ml., and a and b are constants, the linear form of the Langmuir equation is

$$\frac{c}{x/m} = \frac{1}{b}\,c + \frac{1}{a\,b}$$

In this form, the slope is $1/b$ and the intercept is $1/ab$. By plotting $\frac{c}{x/m}$ against c, linear curves are obtained from which may be found the slopes and intercepts. The closer the line is to paralleling c, the greater adsorptive power of the plastic. The slope of the line may be determined, as well as the values of the constants, a and b.

The described procedure provides a satisfactory quantitative method for the evaluation of different plastic materials with respect to their relative adsorptive capacities in contact with various drugs and bacteriostatic and fungistatic agents. Langmuir adsorption isotherms have been employed by Barr, *et al.*, in adsorption studies on clays (8, 9).

The adsorptive avidity of plastic cellulose acetate has been compared with that manifested by plastic cellulose triacetate. As Fig. 3 shows, such avidity with respect to sorbic acid is much higher in case of cellulose acetate. The slopes of the two linear curves for the two respective plastics were calculated and so were the constants a and b. They are shown in Table IV.

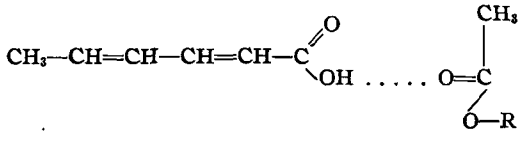
TABLE IV.—CONSTANTS OF LANGMUIR EQUATION FOR SORBIC ACID

Plastic	a	b
Cellulose acetate	0.0085	87.0
Cellulose triacetate	0.0218	20.4

It can be seen that the b value for plastic cellulose acetate is more than four times as high as for cellulose triacetate.

The anhydroglucose units of cellulose each contain three hydroxyl groups, one primary alcohol group and two secondary alcohol groups, which can be acetylated. Study of the distribution of the acetyl groups along the cellulose chain indicates that the esterification is fairly uniform, so that most of the anhydroglucose units will contain at least two acetyl groups. Acetylation obviously alters the surface characteristics of cellulose. The extent of acetylation of cellulose apparently influences the adsorption of weak acidic substances. Vickerstaff suggested that as a consequence of the alteration of the cellulose structure by acetylation, the negative surface potential of cellulose acetate in water is much higher than that of cellulose, and this will assist the repulsion of anionic dyes (10). This theory should extend to cellulose triacetate and sorbic acid. Cellulose triacetate results from the replacement of all or most of the hydroxyl units on the cellulose chain with acetyl groups (11). The much lesser adsorptive capacity of plastic cellulose triacetate as compared with cellulose acetate is in agreement with Vickerstaff's theory.

As has been demonstrated in the experiment with sorbic acid at different pH levels, upon approaching neutrality from the acid side, and nearly complete ionization of the acid, adsorption becomes reduced to zero. The undissociated sorbic acid may be adsorbed on the ester groupings by the formation of the hydrogen bond



The decrease of adsorption upon the increase of the percentage of ionization is not surprising since it seems to have been generally found that ionization of an adsorptive decreases its surface activity. It has been suggested (12) that this may be so because the ion, with its envelope of solvent, is much more soluble than the undissociated molecule, and so shows less tendency to escape from the solution. As is shown in Table III, considerable change in pH occurred at the higher pH values at the end of the experiment. Assays of the controls revealed that no decomposition of the sorbic acid at these high pH levels had occurred. This would indicate that possibly a plasticizer was the cause

of the decrease in pH. The term "apparent equilibration" with reference to the pH data is used advisedly since the data clearly show that the true equilibrium with the original plastic will never be reached. Since the plastic is leaching one or more constituents into the solution, the material is continually being altered and it is improbable that a true equilibrium is attained.

Although the adsorption of acids and bases has been found in some cases to be proportional to the concentration of undissociated molecules, many highly ionized surface-active substances are known so that the extent of ionization itself cannot be a sole controlling factor. As a matter of fact, the experiments conducted with hydroalcoholic solvents have demonstrated the maximum sorbic acid adsorption from the solution containing about 10% (v/v) of ethyl alcohol. This finding had not been anticipated. Autian and Shaikh, in their study on adsorption of sorbic acid by nylon (13), noted that as the water is replaced with a less polar solvent, the binding of sorbic acid decreased. The experiments described, which were carried out three times, show that plastic cellulose acetate behaves differently with respect to sorbic acid. No explanation can be given by this author for the reason or reasons why the adsorption increases and then sharply decreases with increase in alcohol concentration.

The effect of temperature has not been studied because of the instability of sorbic acid at somewhat elevated temperatures (14).

SUMMARY AND CONCLUSIONS

The adsorption of sorbic acid by plastic cellulose acetate and cellulose triacetate has been studied and a method for its determination has been developed. Langmuir adsorption isotherms were determined and the constants calculated. The main significance of this paper is that the applicability of the Langmuir equation to the comparative study of different plastic materials with respect to adsorption has been demonstrated. The method suggested is just one parameter which may be helpful in studying drug–plastic interactions but many other tests and procedures might be necessary to establish useful standards for a particular plastic.

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Multiple Dose Excretion Kinetics

By R. G. WIEGAND, J. D. BUDDENHAGEN, and C. J. ENDICOTT

General mathematical equations describing expected blood and urine drug concentrations were derived to analyze riboflavin excretion data after a single large oral dose followed by multiple smaller but equal doses at equal time intervals. The urinary excretion equation was applied to the riboflavin data to obtain the least squares fit using the IBM digital computer, and by this means the absorption and excretion rates were determined. Such calculations can be applied to find the optimum dosage regimen of other drugs for which the kinetic constants are known. The general expressions reduce to all simpler cases already reported in the literature.

THE FITTING of equations to experimental data on blood and urine concentrations of drug has found wide application in determining the kinetics of the processes involved and in measuring the rates of these processes. Considering absorption and elimination of drug to be apparent first order processes, equations describing blood drug levels (1–4) and urine levels (2, 5) after a single oral dose of drug have been reported. The general equation for blood levels after multiple oral administration of drug at equal doses and time intervals was derived by Dost (4). Boxer, *et al.* (6), had earlier used a simplified form of this equation to obtain the expected maximum and minimum blood levels

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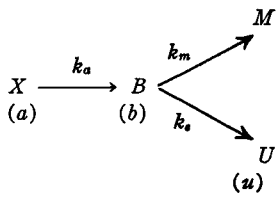
after intravenous administration. For the dosage regimen of an initial large dose and subsequent smaller equal doses at equal time intervals Swintosky, *et al.* (7), have given an approximation for the maximum and minimum blood levels expected, and Krüger-Thiemer (8, 9) has derived the equation giving the ratio of initial to sustaining doses such that the blood level at the time of the first sustaining dose will be maintained.

The general equations describing blood or urine drug levels after administration of an initial dose different in size from successive equal doses at equal time intervals have not been published. Such an equation for blood levels should reduce as time approaches infinity to the expression given by Swintosky, *et al.* (7), for maximum and minimum blood levels, and by appropriate substitutions the dose ratio should be obtained. It should also reduce to the general equations for blood concentration after multiple equal doses or after a single dose. Similarly, the equation for urine levels should reduce to the expression for use with data collected after all equal doses as well as to the equation describing urine drug levels after a single dose. In the process of reducing the general expressions to the simpler forms the assumptions on which the simpler equations are based become apparent and the expected errors resulting from their use can be calculated.

After a large dose and successive smaller doses, the general form of the equations will also make possible calculation of the complete curves for blood or urine levels. This will allow fitting the curves to actual data collected after this dosage regimen, confirming the kinetic order and obtaining a measure of the rates involved. The work with riboflavin reported here was started to find the amount of vitamin necessary in divided doses to maintain the circulating level. The equations were derived to analyze the urinary riboflavin data in terms of the rates of absorption and excretion plus the parameters of dosage.

MATHEMATICAL DERIVATION

After oral administration of a drug, it and its metabolites will pass through a series of compartments according to the flow diagram



where X is the gastrointestinal tract, B is the body exclusive of the gastrointestinal tract and urine, U is the urine, and M indicates the metabolite pool. Apparent first order rate constants are assigned for absorption, k_a, metabolism, k_m, and excretion, k_e. Since when working with blood levels of drug the relative contribution to disappearance of drug by metabolism or excretion is usually not known, the apparent first order disappearance constant, k_d, is defined as the sum of k_e and k_m. The fractional amounts of the total dose in a compartment at any time will be given by the lower case letters in parentheses in the diagram.

Following an initial dose A_o, the amount of drug in the gastrointestinal tract, body, and urine at time τ is given by substitution of $t = \tau$ in previously published equations (5) as

$$a = A_o e^{-k_a \tau} \qquad \text{(Eq. 1)}$$

$$b = \frac{A_o k_a}{k_d - k_a} (e^{-k_a \tau} - e^{-k_d \tau}) \qquad \text{(Eq. 2)}$$

$$u = A_o \frac{k_e}{k_d} \left(1 - \frac{(k_d e^{-k_a \tau} - k_a e^{-k_d \tau})}{(k_d - k_a)}\right) \qquad \text{(Eq. 3)}$$

If a second oral dose, a_o, (different in size from the initial dose) is administered at time τ, the amounts of drug in the compartments a, b, and u can be derived by solving the differential equations

$$-\frac{da}{dt} = k_a a \qquad \text{(Eq. 4)}$$

$$\frac{db}{dt} = k_a a - k_d b \qquad \text{(Eq. 5)}$$

$$\frac{du}{dt} = k_e b \qquad \text{(Eq. 6)}$$

where time, t, is calculated from the time of administration of the last dose. Using Eqs. 1, 2, and 3 for the values of a, b, and u at $t = O$, the equations giving the amount of drug in each compartment as a function of time are

$$a = (a_o + A_o e^{-k_a \tau}) e^{-k_a t} \qquad \text{(Eq. 7)}$$

$$b = \frac{k_a}{k_d - k_a} [(a_o + A_o e^{-k_a \tau}) e^{-k_a t} - (a_o + A_o e^{-k_d \tau}) e^{-k_d t}] \qquad \text{(Eq. 8)}$$

$$u = \frac{k_e}{k_d} \left[A_o + a_o - \frac{k_d(a_o + A_o e^{-k_a \tau})}{k_d - k_a} e^{-k_a t} + \frac{k_a(a_o + A_o e^{-k_d \tau})}{k_d - k_a} e^{-k_d t}\right] \qquad \text{(Eq. 9)}$$

Similar extension of Eqs. 7, 8, and 9 can be made for successive oral doses equal to the second (a_o), administered at the same time interval τ. Under these conditions the term within the parentheses in Eq. 7 becomes $(a_o + a_o e^{-k_a \tau} + a_o e^{-2k_a \tau} + \ldots + a_o e^{-(n-1)k_a \tau} + A_o e^{-nk_a \tau})$. Because the sum of all but the last term in this expression equals $a_o(1 - e^{-nk_a \tau})/(1 - e^{-k_a \tau})$, the expression for a after an initial dose A_o and successive doses a_o given at time intervals τ becomes

$$a = \left(A_o e^{-nk_a \tau} + a_o \frac{1 - e^{-nk_a \tau}}{1 - e^{-k_a \tau}}\right) e^{-k_a t} \qquad \text{(Eq. 10)}$$

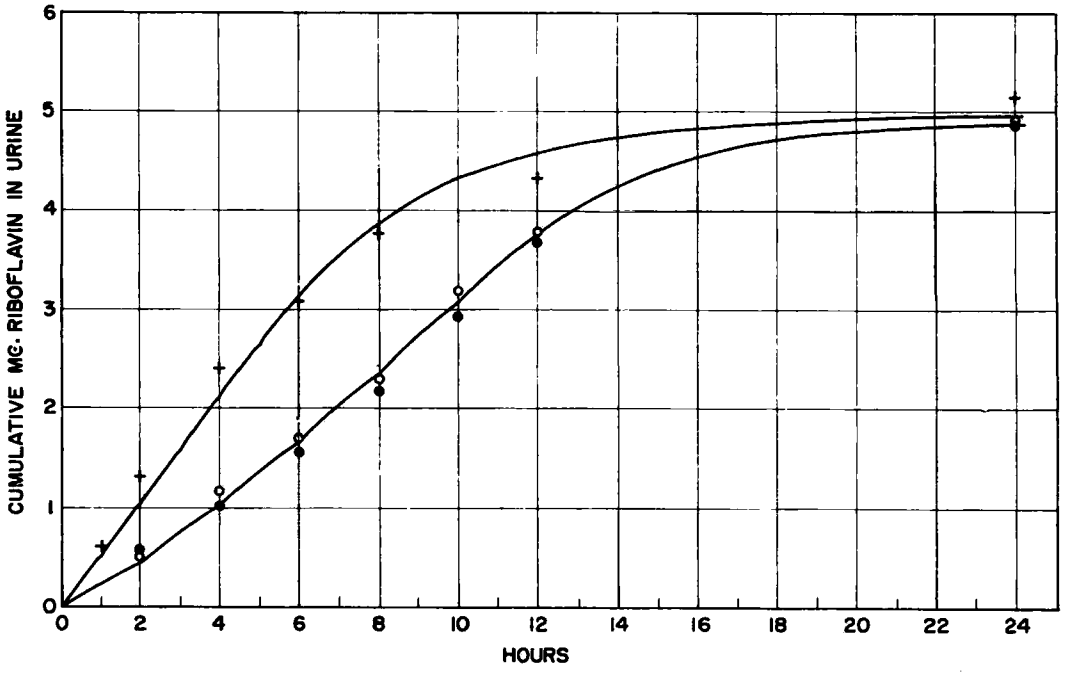


Fig. 1.—Mean cumulative riboflavin urine levels from 10 adults after 5 mg. riboflavin orally at zero time and 5 doses of 1 mg. every hour (+, experiment 800) or after 2.4 mg. at zero time and 5 doses of 1.5 mg. every 2 hours, (○, experiment 801; ●, experiment 996). Curves are calculated according to equation 12 with $k_e = .123$ hr.$^{-1}$, $k_d = .246$ hr.$^{-1}$, $k_a = 30$ hr.$^{-1}$, and appropriate values of A_0, a_0, τ, n, and t.

where n is the number of doses of size a_o. By similar derivations the equations describing b and u in this general case become

$$b = \frac{k_a}{k_d - k_a}\left[\left(A_o e^{-nk_a\tau} + a_o\frac{1 - e^{-nk_a\tau}}{1 - e^{-k_a\tau}}\right)e^{-k_a t} - \left(A_o e^{-nk_d\tau} + a_o\frac{1 - e^{-nk_d\tau}}{1 - e^{-k_d\tau}}\right)e^{-k_d t}\right] \quad \text{(Eq. 11)}$$

$$u = \frac{k_e}{k_d}\left[A_o + na_o - \frac{k_d}{k_d - k_a}\left(A_o e^{-nk_a\tau} + a_o\frac{1 - e^{-nk_a\tau}}{1 - e^{-k_a\tau}}\right)e^{-k_a t} + \frac{k_a}{k_d - k_a}\left(A_o e^{-nk_d\tau} + a_o\frac{1 - e^{-nk_d\tau}}{1 - e^{-k_d\tau}}\right)e^{-k_d t}\right] \quad \text{(Eq. 12)}$$

If the initial dose was the same as all successive doses ($A_o = a_o$) Eq. 11 reduces to the form given by Dost (4) for the case of equal doses administered at equal time intervals

$$b = \frac{a_o k_a}{k_d - k_a}\left[\left(\frac{1 - e^{-n'k_a\tau}}{1 - e^{-k_a\tau}}\right)e^{-k_a t} - \left(\frac{1 - e^{-n'k_d\tau}}{1 - e^{-k_d\tau}}\right)e^{-k_d t}\right] \quad \text{(Eq. 13)}$$

where n' is the number of doses given ($n' = n + 1$). Similar reduction of Eq. 12 can be made, giving

Equation 11 reduces to $b = 0$ at infinite time, which would be expected since the drug is no longer in the body. Equation 12 reduces at infinite time to $u = \frac{k_e}{k_d}(A_o + na_o)$, which indicates that the fraction of the total dosage administered which appears in the urine is a function of the rate of excretion compared to the sum of the excretion and metabolism rates.

According to the nomenclature used above, the fraction of drug in compartment b at the time of administration of the first dose of size a_o is given by substitution of $n = 0, t = \tau$ in Eq. 11. Substitution of $n = \infty, t = \tau$ in the same equation gives the minimum value of b after sufficient doses of size a_o such that the effect of the initial dose A_o is negligible. From these two equations the ratio A_o/a_o can be obtained as

$$\frac{A_o}{a_o} = \frac{1}{(1 - e^{-k_a\tau})(1 - e^{-k_d\tau})} \quad \text{(Eq. 15)}$$

This is the same form as given by Krüger-Thiemer (8) for the ratio of initial dose to supporting dose such that the blood drug levels obtained at time τ after the initial dose will be maintained. The blood levels at any time can be calculated from the expression $c = b/V_d'$, where c = drug concentration in blood (mg./L.) and V_d' = specific volume of distribution (L./Kg.) if dose is in units of mg./Kg., and substituting b from Eq. 11 to give

$$u = a_o\frac{k_e}{k_d}\left[n' - \frac{\{k_d[(1 - e^{-n'k_a\tau})/(1 - e^{-k_a\tau})]e^{-k_a t} - k_a[(1 - e^{-n'k_d\tau})/(1 - e^{-k_d\tau})]e^{-k_d t}\}}{k_d - k_a}\right] \quad \text{(Eq. 14)}$$

$$c = \frac{k_a}{V_d'(k_d - k_a)}\left[\left(A_o e^{-nk_a\tau} + a_o\frac{1 - e^{-nk_a\tau}}{1 - e^{-k_a\tau}}\right)e^{-k_a t} - \left(A_o e^{-nk_d\tau} + a_o\frac{1 - e^{-nk_d\tau}}{1 - e^{-k_d\tau}}\right)e^{-k_d t}\right] \quad \text{(Eq. 16)}$$

METHODS AND RESULTS

Riboflavin U.S.P. in finely divided form was used in this study. Suspensions were freshly prepared in deionized water at a concentration of 10 mg. riboflavin per 50 ml. Each subject drank the appropriate amount of suspension at the indicated interval, rinsing the container with several smaller portions of water to insure consumption of the total dose.

Ten apparently healthy adults were used as subjects. The subjects were placed on a semi-restricted diet, refraining from eggs, liver, and vitamin supplements for one day preceding the test and the two-day test period. On test 996 the diet was more closely controlled. No food, other than a 200-ml. portion of vegetable puree at 2-hour intervals, was consumed during the critical 12-hour Wednesday test period. This test was included to check the effect of diet in the first two experiments where the subjects were allowed to consume the foods of their choice with the exceptions previously mentioned. A 9.9-mg. or 10-mg. dose of riboflavin, divided as outlined in Table I, was used in the study.

The test periods began Tuesday morning at 8:15 and lasted through 8:15 on Thursday morning. On the first day, all the urine accumulated up to 8 hours and from 8 to 24 hours after the start of the test was collected and used to obtain the base riboflavin excretion level for each individual. Wednesday morning at 8:15 a.m. the first dose of the test series was taken. Subsequent doses were taken as indicated in Table I. Urine was collected at the time intervals shown in Table I during each experiment. The urine was collected in amber glass bottles, the volume measured, and aliquots stored at 4° until assayed.

The urine was assayed for riboflavin by the U.S.P. fluorometric method (10). The base value for each individual was subtracted from the riboflavin levels obtained after the ingestion of the test preparation to determine the net amount of riboflavin excreted. The excretion data obtained are presented in Table I.

The data in Table I were analyzed using Eq. 12. In this equation for total amount of drug in urine the parameters A_o, a_o, n, τ, and t are known for any single value of u reported. Because the average fraction of the total dose excreted within 24 hours in the three experiments was 0.50, this value of the ratio k_e/k_d was used. Thus, only k_a and k_e remained to be determined. This was done by assuming values for these constants and evaluating Eq. 12 at each time a urine sample was collected. The sum of the squares of the differences between the observed and calculated values of u was obtained. An IBM 1620 digital computer was used for the calculations. The constants k_a and k_e which minimized this sum of squares were $k_e = 0.123$ hr.$^{-1}$ and $k_a > 30$ hr.$^{-1}$. Values of k_a as great or greater than 30 hr.$^{-1}$ are not meaningful in this application because they indicate that absorption is 95% complete in a tenth of an hour or less.

The mean cumulative urinary riboflavin levels are shown in Fig. 1 for each of the three experiments with the curves calculated according to Eq. 12 with $k_a = 30$ hr.$^{-1}$, $k_e = 0.123$ hr.$^{-1}$, $k_d = 0.246$ hr.$^{-1}$ and other constants as given by Table I.

The theoretical levels of drug in compartment b during the 24-hour duration of the experiment can be calculated from Eq. 11 without further assumptions, and are shown in Fig. 2.

DISCUSSION

The applicability of Eq. 12 to the riboflavin excretion data depends primarily on the assumption of apparent first order kinetics of riboflavin excretion. This kinetic order is substantiated by the riboflavin excretion data of Axelrod, *et al.* (11), and Najjar and Holt (12), obtained after single doses of the drug. First order excretion is also consistent with the finding of Morrison and Campbell (13) that the per cent of dose excreted is constant over the 1 to 20 mg. range of oral doses.

The fraction of the total dose excreted appears from our data to be independent of how the total dose is divided or at what interval the individual doses are administered. This is similar to the findings of Chapman and Campbell (14) and Morrison and Campbell (13), who reported equal recovery of riboflavin in urine after single or divided oral dosage.

TABLE I.—URINARY RIBOFLAVIN EXCRETION IN HUMAN SUBJECTS

Time Interval, hr.	Total Riboflavin (mg.) Excreted During Indicated Interval (Mean ± S.D.)[a] Test 800: 5.0 mg. at $t = o$ and 1.0 mg. every hour for 5 doses	Test 801: 2.4 mg. at $t = o$ and 1.5 mg. every 2 hours for 5 doses	Test 996: 2.4 mg. at $t = o$ and 1.5 mg. every 2 hours for 5 doses
0–1	0.62 ± 0.34		
1–2	0.68 ± 0.20		
0–2		0.53 ± 0.13	0.58 ± 0.13
2–4	1.11 ± 0.31	0.66 ± 0.17	0.45 ± 0.07
4–6	0.70 ± 0.32	0.51 ± 0.19	0.53 ± 0.19
6–8	0.68 ± 0.28	0.61 ± 0.12	0.61 ± 0.14
8–10		0.89 ± 0.18	0.77 ± 0.16
10–12		0.65 ± 0.29	0.73 ± 0.18
8–12	0.56 ± 0.18		
12–24	0.60 ± 0.11	1.13 ± 0.36	1.23 ± 0.33

[a] Each value is based on the mean of 10 subjects.

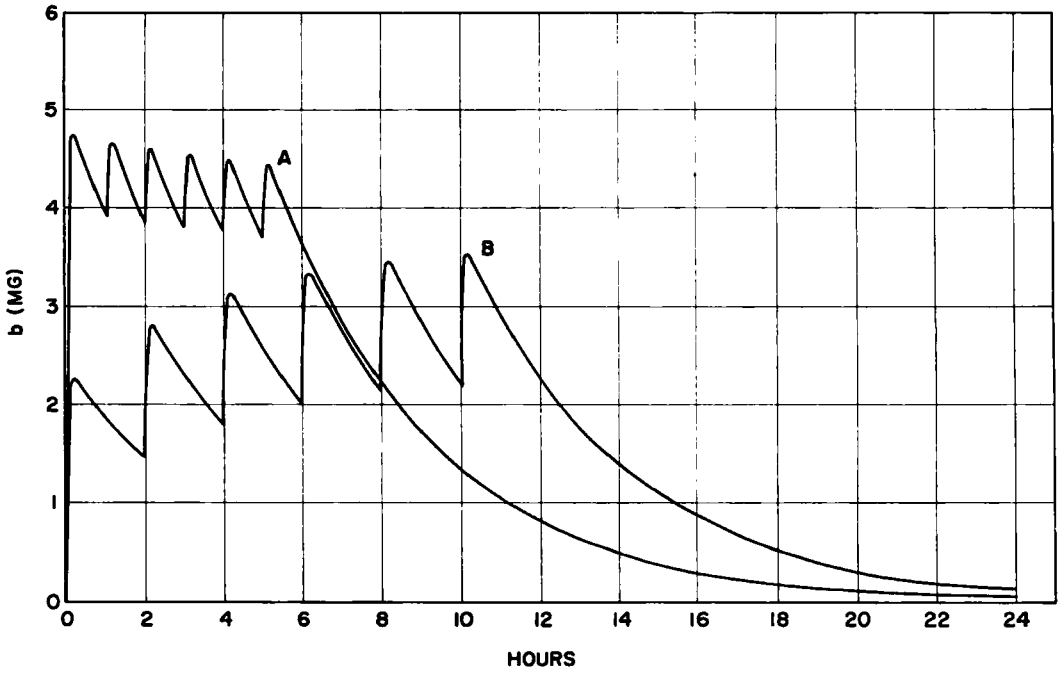


Fig. 2.—Amount of riboflavin in the body (b) as calculated from Eq. 11 with k_d = .246 hr.$^{-1}$ and k_a = 30 hr.$^{-1}$. In curve A, A_0 = 5 mg., a_0 = 1 mg., τ = 1 hr., and $n_{max.}$ = 5. In curve B, A_0 = 2.4 mg., a_0 = 1.5 mg., τ = 2 hr., and $n_{max.}$ = 5.

The shape of the excretion curve calculated from Eq. 12 during any time period τ is convex, rising more rapidly during the first part of the period than later. This is because riboflavin appears to be rapidly absorbed, causing higher blood levels early in the period (see Fig. 2), and thus more rapid excretion than occurs toward the end of the interval. For drugs which are slowly absorbed the shape of the excretion curve would be sigmoid, with the inflection occurring at the time of peak blood concentration.

More apparent in this type experiment than the shape of the excretion curve in the interval τ is the shape over the period of the whole experiment. If blood levels of drug are higher after each successive dose (due to a low dose ratio, A_0/a_0) the general shape of the excretion curve will be concave, rising more steeply during each successive interval. This is the shape of the lower curve in Fig. 1 where the dose ratio was 1.6. If blood levels of drug are lower after each successive dose due to a high dose ratio, a generally convex excretion curve will result. This is the case in test 800 in which the dose ratio was 5.0.

Absorption rate, as well as kinetic order, is best determined by the shape of the curve in the time period in which absorption is occurring. In these experiments urine samples over the period of drug administration were emphasized rather than consecutive samples after a single administration. Thus, the shape of the curves obtained from the data in Table I are primarily determined by the dose ratio and the time interval between doses. This does not mean that absorption rate cannot be calculated from this type experiment. In theory it can be calculated under any combination of dosage variables, but in practice a precise measure is best obtained using Eq. 12 when absorption is not essentially complete in the interval between doses.

The equations derived for blood and urine drug levels after an initial dose different from the remaining doses reduce to the forms given in the literature for more specific cases. Equation 13, which gives the values of b after all equal doses at equal time intervals, is the same as that of Dost (4) except for nomenclature. This equation, for the case of intravenous administration ($k_a = \infty$) considered by Boxer, *et al.* (6), simplifies to $b = a_0(1 - e^{-n'k_d\tau})\, e^{-k_d t}/(1 - e^{-k_d\tau})$. Neglecting distribution of drug immediately after injection, the ratio of the value of b when equilibrium is established, b_∞, ($n' = \infty$), to its value in the first period, b_1, ($n' = 1$), is $b_\infty/b_1 = 1/(1 - e^{-k_d\tau})$. This is the expression given by Boxer, *et al.*

Considering the more cymplicated case in which the initial dose is different than successive doses and in which drug is administered orally, the blood levels (c in Eq. 16) or fraction of drug in the body (b in Eq. 11) after a large number of doses such that equilibrium is established can be calculated from the equations after substitution on $n = \infty$ and simplifying. Thus, Eq. 16 simplifies to the form of Eq. 13 and is similar to the treatment of Boxer, *et al.* (6), except that k_a is not large enough to make the exponential terms containing it zero. Maximum and minimum values of blood concentration are then given by the equations

$$c_{max.} = \frac{a_0 k_a}{V_d'(k_d - k_a)} \left[\frac{e^{-k_a t_{max.}}}{1 - e^{-k_a\tau}} - \frac{e^{-k_d t_{max.}}}{1 - e^{-k_d\tau}} \right] \quad \text{(Eq. 17)}$$

where

$$t_{max.} = ln \frac{\left[\frac{k_d(1 - e^{-k_a\tau})}{k_a(1 - e^{-k_d\tau})}\right]}{k_d - k_a} \quad \text{(Eq. 18)}$$

and

$$c_{min.} = \frac{a_o k_a}{V_d'(k_d - k_a)} \left[\frac{e^{-k_a\tau}}{1 - e^{-k_a\tau}} - \frac{e^{-k_d\tau}}{1 - e^{-k_d\tau}}\right] \quad \text{(Eq. 19)}$$

Equations 17 and 18 are identical to the equations derived by Dost (4) for all equal doses because as n approaches infinity the effect of the initial dose on equilibrium blood levels disappears. Equation 19 differs from the equation given by Dost for minimum blood levels only by the exponential factors in the numerators, and reduces directly to the form given by Dost. Equation 19 also reduces to a form similar to the expression given by Swintosky, *et al.* (7), for minimum blood levels when k_a is large. However, Swintosky uses one extrapolated value for $a_o k_a / V_d'(k_d - k_a)$ into which he also combines the absorption terms. Swintosky arrives at his equation for $c_{max.}$ by similar means.

The dose ratio, A_o/a_o, which will maintain a minimum blood level equal to that existing at time τ after the first dose, A_o, is given in Eq. 15. This has been given by a different derivation by Krüger-Thiemer (8), who suggested its clinical application for cases in which k_a is large and $\tau = t_{1/2}$, the half-life of drug disappearance, whereby the dose ratio becomes 2. When k_a is large such that absorption is complete in the interval τ, the equilibrium minimum blood level obtained after many doses is also obtained after the first dose, resulting in a plateau effect (15). From Eq. 16 the effect of repeated doses before absorption of the previous dose is complete is found to be an increase in both maximum and minimum blood levels above the equilibrium values. These higher blood concentrations decrease to the equilibrium values when n becomes large. Thus the dose ratio given by Krüger-Thiemer will not give the plateau effect desired if the product $k_a\tau$ is not large, although minimum drug concentrations will be maintained.

The equations given above should be useful in the interpretation of blood drug concentration or cumulative drug urine level data obtained after the common dosage regimen of one large dose and subsequent equal smaller doses at equal time intervals. Such experiments are useful in determining that the calculated dosage regimen is optimal, and the analysis of the results according to the appropriate equation should allow an assessment of what actually occurred. The equations in their general form are also useful for the derivation of simpler forms by substitution of the appropriate limiting assumptions.

SUMMARY

1. General mathematical equations have been derived to analyze plasma concentration or urinary excretion data obtained when a drug is given as one large dose followed by successive equal small doses at equal time intervals.

2. The validity of the urinary excretion equation has been demonstrated by fitting the equation to urinary excretion data from human subjects. Assuming first-order kinetics, the absorption, disappearance, and excretion constants of riboflavin were found to be: $k_a = 30$ hr.$^{-1}$, $k_d = 0.25$ hr.$^{-1}$, and $k_e = 0.12$ hr.$^{-1}$.

3. The disappearance rate was found to be the controlling factor for sustaining riboflavin levels. In the case of riboflavin, absorption is almost immediate and an accurate measure of the absorption rate was not made.

4. Expected maximum and minimum blood levels can be calculated when the necessary rate constants and volume of distribution are known.

5. The equations presented reduce to simpler expressions found in the literature by substituting appropriate limiting functions.

6. These equations allow calculation of the ratio of the initial dose to subsequent smaller doses given at equal time intervals which will maintain a relatively constant blood level. Such calculations can be applied to find the optimum dosage regimen of any drug for which the absorption, excretion, and disappearance constants are known.

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Spectrophotometric Analysis of Phenobarbital and Pentobarbital in Pharmaceuticals

By THEODORE L. BROWN

An analytical procedure has been developed for measuring the concentration of phenobarbital and pentobarbital in mixtures of these substances, *e.g.*, in pharmaceuticals. This new method is a spectrophotometric procedure based upon the shift in ultraviolet absorbance of pentobarbital subsequent to dealkylation in sulfuric acid.

IN THE COURSE of their investigation of the structure of the metabolites of pentobarbital, Maynert and Washburn (1) found that certain dialkyl barbituric acids are subject to dealkylation in sulfuric acid. These workers reported that 5,5-dialkyl barbituric acids containing a secondary group lost this group in sulfuric acid. For example, 5-ethyl-5-(1-methylbutyl)-barbituric acid was dealkylated to 5-ethyl barbituric acid. Monoalkyl derivatives, such as 5-(1-methylbutyl)-barbituric acid, were found to be stable in this menstruum, as were 5,5-disubstituted barbituric acids containing two primary alkyl groups or a phenyl and a primary alkyl group.

Brooker (2) found this reaction mechanism could be utilized in the analysis of mixed barbiturates by paper chromatography. He demonstrated that the chromatographic resolution of amylobarbital and pentobarbital could be markedly improved by selectively converting the pentobarbital to 5-ethyl barbituric acid with hot sulfuric acid.

Curry (3) reported that the ultraviolet absorbance maximum of pentobarbital shifts from 240 to 268 millimicrons as a consequence of dealkylation. He found that phenobarbital under these conditions was completely destroyed and the ultraviolet absorbance of this substance became zero as a consequence of this hot acid treatment.

Work in this laboratory has confirmed the shift in the ultraviolet absorbance maximum of pentobarbital. However, we found that the absorbance of phenobarbital is essentially unchanged by hot sulfuric acid treatment (see Fig. 1). These results are consistent with Maynert and Washburn's observations concerning the stability of 5,5-disubstituted barbituric acids in sulfuric acid solution

These data suggested the possibility of using this technique for measuring the concentration of both phenobarbital and pentobarbital in pharmaceuticals containing both of these substances.

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EXPERIMENTAL

In order to define the necessary conditions for the proposed analytical technique, the following experiments were performed:

Effect of Heating Time On Dealkylation of Pentobarbital.—An aqueous solution was prepared by dissolving 25.0 mg. of pentobarbital in 5.0 ml. of 0.13 *N* sodium hydroxide. One-milliliter aliquots were mixed with 9.0 ml. of concentrated sulfuric acid and heated at 100° for increments of time. Subsequent to cooling, these samples were diluted to 50.0 ml. with distilled water. Five-milliliter aliquots were mixed with 5.00 ml. of 22.5% ammonium hydroxide and then diluted to 100.0 ml. with distilled water. The absorbance of these solutions at 240 and 268 mμ was then measured, using a Beckman DU spectrophotometer. The results are recorded in Table I. It would seem from these data that timing of the acid treatment step is not critical. Thirty minutes at 100° was used for all subsequent work.

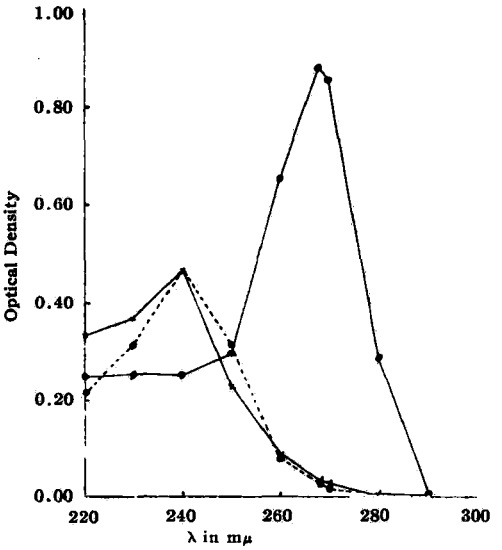

Fig. 1.—U.V. absorbance of phenobarbital and pentobarbital before and after treatment with hot sulfuric acid: ⊙-⊙-⊙ 10 mcg. pentobarbital/ml. before hot sulfuric acid treatment; ⊙—⊙—⊙ 10 mcg. pentobarbital/1 ml. after hot sulfuric acid treatment; ×—×—× 10 mcg. phenobarbital/ml. before and after hot sulfuric acid treatment.

TABLE I.—EFFECT OF HEATING TIME ON PENTOBARBITAL DEALKYLATION

Time of Heating, min.	Absorbance 240mμ	Absorbance 268mμ
0	0.466	0.022
15	0.269	0.846
30	0.252	0.880
60	0.252	0.880

Effect of Sulfuric Acid Concentration on Rate of Dealkylation of Pentobarbital.—An aqueous solution was prepared by dissolving 25.0 mg. of pentobarbital in 5.0 ml. of 0.13 *N* sodium hydroxide. One-milliliter aliquots were mixed with 9.0 ml. of sulfuric acid of varying strengths. Subsequent to cooling, these samples were diluted to 50.0 ml. with distilled water. Five-milliliter aliquots were mixed with 5.0 ml. of 22.5% ammonium hydroxide and then diluted to 100.0 ml. with distilled water. The absorbances were then measured at 240 and 268 mμ. The results are recorded in Table II.

TABLE II.—EFFECT OF ACID CONCENTRATION ON PENTOBARBITAL DEALKYLATION

Concentration of Acid Added	Absorbance 240mμ	Absorbance 268mμ
21 *N*	0.445	0.018
30 *N*	0.431	0.102
36 *N*	0.252	0.880

It is apparent from these data that acid concentration is critical and must be carefully controlled. In all of the subsequent work, 36 *N* sulfuric acid was used in the analysis.

Interfering Substances.—Only those substances present in the pharmaceutical preparation of interest in this laboratory were evaluated with respect to their effect on the proposed analytical technique. It was found that preliminary purification by filtration and solvent extraction eliminated most of these substances prior to acid treatment. Thus, lactose, calcium stearate, procaine hydrochloride, and N-ethyl-3-piperidyl benzilate methobromide were found to be without significant effect on the proposed barbiturate analysis.

Effect of pH on Ultraviolet Absorbance of Phenobarbital, Pentobarbital, and Ethyl Barbituric Acid.—It is known that pH has significant effect on ultraviolet absorbance of barbiturates and their derivatives. However, it is extremely simple to prepare samples of exactly reproducible pH by mixing measured volumes of reagents of known concentration. Our experience indicated that if ordinary quantitative techniques are observed in measuring the standardized reagents employed, the variation in absorbance on a given sample will be negligible.

Summation of Experimental Procedure—Ten milliliters of an ethereal extract containing 2.5–5.0 mg. each of phenobarbital and pentobarbital was transferred to a 2.2 × 24 cm. test tube. The ether was carefully evaporated off with the aid of a warm water bath. The residual barbiturates were then dissolved in 1.0 ml. of 0.13 *N* sodium hydroxide and mixed with 9.0 ml. of 36 *N* sulfuric acid. This mixture was heated at 100° for 30 minutes and then cooled to RT. The sample was then placed in an ice bath and diluted to 50.0 ml. with distilled water. Five milliliters of this solution was mixed with 5.0 ml. of 22.5% ammonium hydroxide and then diluted to 100.0 ml. with distilled water. The absorbance was then measured against a reagent blank at 240 and 268 mμ.

TABLE III.—EFFECT OF HOT SULFURIC ACID TREATMENT UPON ULTRAVIOLET ABSORBANCE OF PHENOBARBITAL AND PENTOBARBITAL

Wavelength, mμ	Absorbance of Phenobarbital[a] Heated	Absorbance of Phenobarbital[a] Unheated	Absorbance of Pentobarbital[a] Heated	Absorbance of Pentobarbital[a] Unheated
220	0.336	0.336	0.244	0.216
230	0.366	0.366	0.254	0.309
240	0.465	0.466	0.252	0.466
250	0.220	0.222	0.296	0.308
260	0.082	0.083	0.656	0.078
268	0.032	0.031	0.880	0.022
270	0.022	0.021	0.856	0.015
280	0.003	0.003	0.282	0.001
290	0.000	0.000	0.006	0.000

[a] Concentration of each barbiturate is 10.0 mcg./ml.

Pure samples of phenobarbital and pentobarbital were analyzed by this technique and the absorbances were measured at intervals throughout the ultraviolet spectrum. Similar samples were prepared and the absorbances similarly measured after addition of the acid but prior to heat treatment. These data are recorded in Table III. These data were then used to calculate the concentration in unknown sample mixtures as follows: Let X = mcg. of pentobarbital/ml., and Y = mcg. of phenobarbital/ml. Then, $0.465\ Y/10 + 0.252\ X/10$ = absorbance at 240 mμ and $0.032\ Y/10 + 0.880\ X/10$ = absorbance at 268 mμ.

Substituting in the absorbance values observed on a given sample and then solving simultaneously, one can determine the concentration of each of the barbiturates in the dilute ammoniacal solution. Back calculating by substituting in the appropriate dilution factors, one can determine the concentration of each of the barbiturates in the original sample.

RECOVERY EXPERIMENT

Mixtures were prepared containing varying proportions of phenobarbital and pentobarbital. Aliquots of the resultant solutions were analyzed in the proposed manner. The results obtained are recorded in Table IV.

TABLE IV.—PER CENT RECOVERY OF PHENOBARBITAL AND PENTOBARBITAL IN KNOWN MIXTURES

Sample	Phenobarbital, mcg./ml. Theor.	Found	% Recovery	Pentobarbital, mcg./ml. Theor.	Found	% Recovery
1	6.04	6.01	99.7	4.02	4.08	101.3
2	5.04	5.04	100.0	5.02	5.04	100.1
3	4.03	4.09	101.4	6.02	6.11	101.3

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Chemistry of Guareschi Imides I

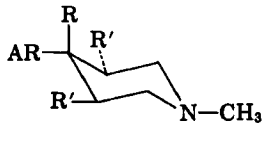
Lithium Aluminum Hydride Reduction of Some Bicyclo Guareschi Imides

By ARNOLD A. LIEBMAN† and FRANK E. DiGANGI

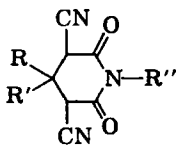
In the course of the synthesis of some 3,5-disubstituted piperidine compounds which would also contain the essential requirements for analgesic activity, some Guareschi imides were prepared and their subsequent reduction with lithium aluminum hydride was studied.

IN AN EFFORT to prepare compounds of types I, II, and III as potential analgesics, Guareschi imides (IV) were synthesized as model compound intermediates. If lithium aluminum hydride would selectively reduce the imide grouping, then the intermediate dicyanopiperidines (V) could be converted into the desired compounds (I, II) and total reduction of these imides should yield III. A review of the literature shows no attempted selective reduction of cyanoimides. Avison and Morrison (1) have successfully reduced ethyl-5-chloro-2-cyano-2-phenylvalerate (VI) to 5-chloro-2-cyano-2-phenyl-1-pentanol (VII) but the lactam (VIII) derived from VII yielded only a mixture of basic products when selective reduction was attempted.

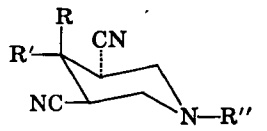
The model β,β-dialkyldicyanoimides (IV) were prepared using the modified procedure of Benica and Wilson (2) for the Guareschi reaction (3). It was noted that the Guareschi imides are insoluble in diethyl ether and other solvents commonly used in lithium aluminum hydride reductions, being only slightly soluble in tetrahydrofuran. Therefore, this solvent was used exclusively in the reduction studies. Selective reduction of the Guareschi imides was attempted under various conditions. Dissolving the imide in the minimum amount of peroxide-free tetrahydrofuran and adding a calculated amount of lithium aluminum hydride dissolved in tetrahydrofuran resulted in an immediate precipitate. For example, treating α,α'-dicyano-β,β-dimethyl-N-methylglutarimide (IX) with a molecular equivalent of lithium aluminum hydride resulted in the formation of a precipitate. The mixture was not heated but decomposed by adding water until refluxing had ceased. The solid material obtained by filtering the mixture was dissolved in hydrochloric acid and after extraction with ether, the solution alkalinized with sodium carbonate. After filtering the



I AR—C_6H_5 R—alkyl
R′—CH_3CH_2OOC—
II AR—C_6H_5 R—alkyl
R′—CH_3CH_2CO—
III AR—C_6H_5 R—alkyl
R′—H_2NCH_2—

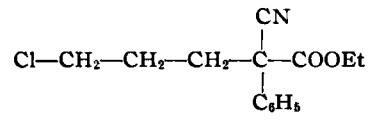


IV
R,R′—alkyl
R″—H, CH_3

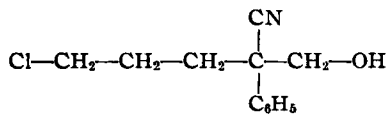


V

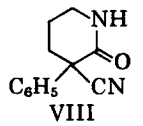
R,R′—alkyl
R″—H, CH_3



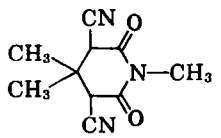
VI



VII



VIII



IX

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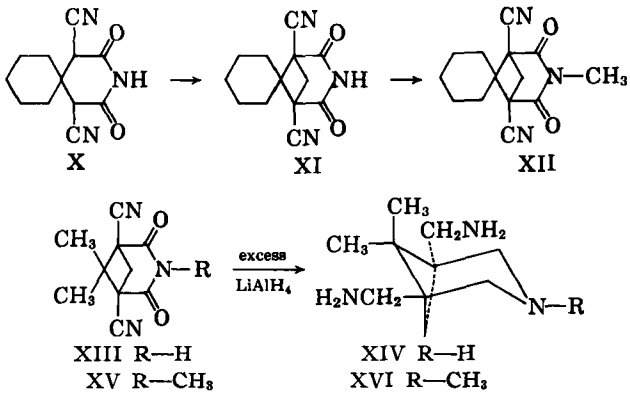


resulting suspension followed by extraction with ether, evaporation of the ether left a small amount of solid material. This material melted higher than starting material and an infrared spectrum showed the retention of carbonyl and cyano functions. The organic solution from the reaction mixture deposited only starting material when evaporated. The original aqueous layer was evaporated to a small amount of impure semisolid, the quality of which precluded any attempts at characterization.

In addition, selective reduction was attempted allowing the reaction mixture to reflux for periods of from 2–24 hours and the complex being decomposed with either acid or alkali, extracting the organic material with suitable solvents. Also, increasing amounts of lithium aluminum hydride were used. In each instance, however, evaporation of the solvent left a small amount of hygroscopic semisolid but no pure reduction product could be isolated. Regardless of the compound or the experimental conditions, similar results were encountered.

It became apparent that the lithium aluminum hydride easily complexed with the acidic hydrogen atoms of the Guareschi imides and the resulting tetrahydrofuran insoluble material does not undergo the desired reduction. The preparation of XII[1] represents a compound having no acidic hydrogen. This compound was prepared by first synthesizing the Guareschi imide (X) from cyclohexanone, ethylcyanoacetate, and alcoholic ammonia. The bicyclo compound (XI) was prepared by the method of Kerr (4) and Paul (5), using sodium methoxide and methylene iodide. Methylation of the imide nitrogen was effected with sodium hydride and methyl iodide. Attempted selective reduction of this compound (XII) proceeded in a manner similar to the monocyclic compounds and gave similar results in that no pure reduction product could be identified.

Total reduction of the β,β-dialkyldicyanoglutarimides was attempted by adding a solution of the imide in tetrahydrofuran to an excess of lithium aluminum hydride contained in an ether-tetrahydrofuran mixture. Again, a precipitate formed, and regardless of the conditions used, no appreciable amounts of reduced material could be obtained.

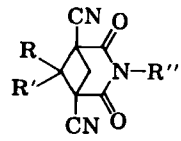
Total reduction of a bicyclo Guareschi imide was attempted under the same conditions with the expected diaminomethylpiperidine being obtained. The Guareschi imide derived from acetone was converted into a bicyclo compound (XIII) with methylene iodide and subsequent reduction yielded 20% of the expected product (XIV). The N-methyl analog (XV) yielded 38% of the diaminomethyl-N-methylpiperidine (XVI) upon reduction.

Two unsymmetrical Guareschi imides were prepared to determine their susceptibility toward bicyclization with methylene iodide and subsequent reduction with lithium aluminum hydride. The Guareschi imide derived from 2-butanone was readily prepared. Due to the extremely low yield of Guareschi imide derived from 4-1 ethyl-2-pentanone in the Guareschi synthesis (6), this compound was prepared in an overall yield of 54% using the two step procedure for the preparation of β-alkyl-β-aryl Guareschi-like imides (7–9). These two Guareschi imides along with the two previously mentioned readily underwent bicyclization with methylene iodide yielding the products shown in Table I, Nos. 1–4. The sodio derivatives of the various bicyclo Guareschi imides were prepared with sodium hydride in tetrahydrofuran and each underwent N-methylation with methyl iodide (Table I, Nos. 5–8).

The general procedure for reduction of the bicyclo imides was patterned after the successful

[1] Named according to the Ring Index, compound XII is: spiro-(3-aza-1,5-dicyano-2,4-dioxo-3-methyl-bicyclo-(3.1.1)-heptane-6,1′-cyclohexane).

TABLE I.—DATA FOR PREPARED COMPOUNDS



No.	R	R′	R″	Formula	M.p.,[a] °C.	Recrystallizing Solvent	Yield, %	Carbon, % Calcd.	Carbon, % Found[b]	Hydrogen, % Calcd.	Hydrogen, % Found
1	CH_3	CH_3	H	$C_{10}H_8N_2O_2$	319[c]	Methanol-acetone	46	...	...	...	...
2	CH_3	CH_3CH_2	H	$C_{11}H_{10}N_2O_2$	242–244	Ethanol	56	60.82	61.00	5.11	5.18
3	CH_3	$(CH_3)_2CHCH_2$	H	$C_{13}H_{16}N_2O_2$	243–245	Ethanol	66	63.66	63.66	6.16	6.13
4			H	$C_{12}H_{12}N_2O_2$	257–264[d]	Methanol	66	...	...	...	...
5	CH_3	CH_3	CH_3	$C_{11}H_{10}N_2O_2$	229–231	Absolute ethanol	85	60.82	60.67	5.11	5.16
6	CH_3	CH_3CH_2	CH_3	$C_{12}H_{12}N_2O_2$	174	Ethanol	55	62.32	62.48	5.66	5.74
7	CH_3	$(CH_3)_2CHCH_2$	CH_3	$C_{14}H_{17}N_2O_2$	204–208	Ethanol	98	64.85	65.29	6.61	6.78
8			CH_3	$C_{14}H_{16}N_2O_2$	247–249	Ethanol	87	65.35	65.53	5.88	6.00

[a] Melting points are uncorrected and were taken with a Thomas Hoover capillary tube melting point apparatus. [b] Analyses were performed by the Microanalytical Laboratory of the University of Minnesota. [c] Melts with decomposition. Reported (4) m.p., 305–306° (decompn.). [d] Reported (5) m.p., 266°.

reduction of 3-aza-1,5-dicyano-2,4-dioxo-6,6-dimethyl-bicyclo-(3.1.1)-heptane and its N-methyl analog (Nos. 1 and 5, Table I). It was found that a 30% excess of lithium aluminum hydride over the theoretical amount and a reflux time of 9 hours gave the best yields (Table II). The N-methyl-bicyclo-glutarimides gave slightly higher yields on reduction than those possessing the somewhat acidic free imide. The resulting compounds were extremely hygroscopic as were derivatives prepared from them. The purity of these compounds was established by elemental analysis of the base or of its picrate derivative and gas chromatograms. Compounds 11 and 15 (Table II) analyzed for tripicrate derivatives while compound 14 (Table II) appears to have formed a mixture of di- and tripicrate derivatives.

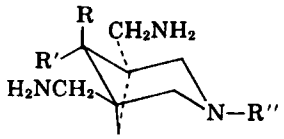
EXPERIMENTAL

General procedure for preparation of Guareschi Imides (2).—The appropriate ketone (0.5 mole), 1.1 mole of freshly distilled ethyl cyanoacetate, and 225 ml. of alcoholic ammonia were mixed and refrigerated for 3 days in a well-stoppered flask. The precipitated ammonium salt was collected and, after washing with ether, dissolved in approximately 350 ml. of warm water. Sufficient concentrated hydrochloric acid was added to insure complete precipitation and the imide then recrystallized from dilute alcohol.

The ketones used in this procedure were acetone, 2-butanone, and cyclohexanone.

Ethyl - (1 - isobutylethylidene) - cyanoacetate.—A mixture of 79.1 Gm. (0.07 mole) of ethyl cyanoacetate, 70.0 Gm. (0.7 mole) of 4-methyl-2-pentanone, 33.6 Gm. (0.56 mole) of glacial acetic acid, 10.8 Gm. (0.14 mole) of ammonium acetate, and 140 ml. of anhydrous benzene were placed in a flask fitted with a Dean-Stark water separator and

TABLE II.—DATA FOR PREPARED COMPOUNDS



No.	R	R′	R″	Formula	B.p. or m.p.,[a] °C.	Yield, %	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
9	CH_3	CH_3	H	$C_{10}H_{21}N_3$	109–110/0.16 mm.	20	65.52	65.25	11.55	11.61
10	CH_3	CH_3CH_2	H	$C_{11}H_{23}N_3$	128–130/0.8 mm.	16	...	...	...	...
		tripicrate		$C_{29}H_{32}N_{12}O_{21}$	245–248[b]		39.37	39.63	3.65	3.77
11	CH_3	$(CH_3)_2CHCH_2$	H	$C_{13}H_{27}N_3$	136/0.3 mm.	10	69.28	69.02	12.08	11.90
12			H	$C_{13}H_{25}N_3$	155/0.45 mm.	23	69.90	69.92	11.28	11.30
13	CH_3	CH_3	CH_3	$C_{11}H_{23}N_3$	94–95/0.15 mm.	38	66.96	67.01	11.75	11.66
14	CH_3	CH_3CH_2	CH_3	$C_{12}H_{25}N_3$	115/0.4 mm.	29	68.19	69.34	11.92	11.84
		dipicrate		$C_{24}H_{29}N_9O_{14}$	209–210[b]		43.05	41.31	4.67	4.72
		tripicrate		$C_{30}H_{34}N_{12}O_{21}$			40.09	"	3.81	"
15	CH_3	$(CH_3)_2CHCH_2$	CH_3	$C_{14}H_{29}N_3$	125–127/0.5 mm.	47	70.23	68.60	12.21	12.15
		tripicrate		$C_{32}H_{38}N_{12}O_{21}$	205–207[b]		41.47	41.42	4.13	4.41
16			CH_3	$C_{14}H_{27}N_3$	138–140/0.5 mm.	21	70.83	70.48	11.46	11.74

[a] All reported boiling points are uncorrected. [b] Melts with decomposition.

treated according to the procedure of Cope (7). Distillation yielded 101.6 Gm. (72%) of the product boiling between 90–95°/0.6 mm.

α,α′ - Dicyano - β - isobutyl - β - methylglutarimide.—According to the procedure of McElvain and Clemens (8, 9), 0.5 mole of sodium cyanoacetamide was treated with 0.5 mole of ethyl-(1-isobutylethylidene)-cyanoacetate. The product was dried to a constant weight of 87.3 Gm. (75%) and melted at 237–239°. Reported (6) m.p. 239–240°.

Selective Reduction Studies.—In a 1-L., three necked, round-bottom flask fitted with a stirrer, dropping funnel, condenser, and drying tube was placed a solution of 10.3 Gm. (0.05 mole) of α,α′-dicyano - β,β - dimethyl - N - methylglutarimide dissolved in 100 ml. of absolute tetrahydrofuran. The flask was cooled in an ice bath and a solution of 1.9 Gm. (0.05 mole) of lithium aluminum hydride dissolved in tetrahydrofuran[2] was added with stirring over a period of 1 hour. A solid began to separate after approximately one-half of the hydride solution was added. After the addition was completed, stirring was continued for 15 minutes at room temperature and then 100 ml. of water was cautiously added. The solid material was filtered with suction and washed with water. Five hundred milliliters of ether was added to the filtrate and the two layers separated. Evaporation of the aqueous layer under reduced pressure yielded a small amount of highly colored semisolid which could not be purified. The organic layer, when evaporated, left starting material only.

The solid obtained during the decomposition of the reaction mixture was dissolved in an excess of 6 *N* hydrochloric acid and the resulting solution extracted with ether. Upon evaporation of the ether, an additional small amount of starting material was obtained. The acidic solution was neutralized with sodium bicarbonate and the precipitated aluminum compounds filtered with suction. The filter cake was washed with ether and these washings were then used to extract the now alkaline solution. After drying the ethereal extract with anhydrous sodium sulfate, the solvent was evaporated under reduced pressure, leaving approximately 0.5 Gm. of a white solid. This material was recrystallized several times from 50% ethanol and melted at 206°. The infrared spectrum of this compound showed retention of carbonyl and nitrile functions.

Selective reduction was also attempted under the experimental conditions of increasing reflux periods up to 24 hours and/or decomposing the hydride complex with 10% sulfuric acid (10). In addition, increasing amounts of lithium aluminum hydride, up to 100% excess, were used. In each instance, the apparent reduction product(s) consisted of highly colored, ether-insoluble semisolid material. Typical amine salts such as methiodide, hydrochloride, citrate, etc., were prepared from tetrahydrofuran solution, but were of such a hygroscopic nature that purification could not be effected. Attempts to purify the reduced material by microdistillation *in vacuo* were unsuccessful in that the material resinified.

The imides used in these studies were α,α′-dicyano-β,β-dimethylglutarimide, α,α′-dicyano-β,β-dimethyl-N-methylglutarimide, and spiro-(3-aza-1,5-dicyano-2,4-dioxo-3-methyl-bicyclo-(3.1.1)-heptane-6,1′-cyclohexane).

Total Reduction Studies.—A suspension of 15.5 Gm. of 95% lithium aluminum hydride (0.39 mole) in a mixture of 150 ml. of absolute ether and 50 ml. of absolute tetrahydrofuran was heated under reflux for 1 hour to effect solution of the hydride. After cooling, 15.3 Gm. (0.08 mole) of α,α′-dicyano-β,β-dimethylglutarimide dissolved in 350 ml. of absolute tetrahydrofuran was added dropwise with stirring. When the addition was completed, the reaction mixture was stirred and heated under reflux for 7 hours during which time a thick, dark yellow precipitate appeared. The mixture was cooled and decomposed by cautiously adding 16 ml. of water, 16 ml. of 15% sodium hydroxide solution, and 48 ml. of water. The gelatinous precipitated material was removed by filtration and washed with two 50-ml. portions of tetrahydrofuran. The combined filtrates, which had a yellow color, were dried (anhydrous sodium sulfate) and then evaporated under reduced pressure. The residue consisted of 3.6 Gm. of starting material and approximately 100 mg. of a dark yellow oil having amine characteristics. A stable solid derivative could not be prepared from this oil.

This reduction was also attempted using 50% excess lithium aluminum hydride in addition to the theoretical amount required to reduce the imide and two nitrile groups. The reflux time was increased to 24 hours but no change in the results was noted. Similar results were obtained in the attempted total reduction of α,α′-dicyano-β,β-dimethyl-N-methylglutarimide.

3-Aza-1,5-dicyano-6,6-dimethyl-2,4-dioxo-bicyclo-(3.1.1)-heptane.[3]—Following the procedure of Kerr (4) and Paul (5), 61.2 Gm. (0.32 mole) of α,α′-dicyano-β,β-dimethylglutarimide was converted into its trisodium salt and treated with 129.0 Gm. (0.048 mole) of methylene iodide. The crude product recovered from aqueous acid weighed 30.0 Gm. (46%) and melted between 305–315° (decompn.). When recrystallized from methanol and acetone, the melting point was raised to 319° (decompn.). Reported (4) m.p. of the product recrystallized from glacial acetic acid is 305–306° (decompn.).

3-Aza-1,5-dicyano-2,4-dioxo-3,6,6-trimethyl-bicyclo-(3.1.1)-heptane.—Sodium hydride, 6.6 Gm. of a 54.5% dispersion (0.15 mole), was suspended in 100 ml. of absolute tetrahydrofuran contained in a 1-L., three-necked, round-bottom flask equipped with a stirrer, dropping funnel, reflux condenser, and drying tube. To the suspension, 30.0 Gm. (0.15 mole) of 3-aza-1,5-dicyano-6,6-dimethyl-2,4-dioxo-bicyclo-(3.1.1)-heptane dissolved in 300 ml. of absolute tetrahydrofuran was added dropwise with stirring. The reaction mixture was refluxed for 1 hour, cooled, and 24.0 Gm. (0.17 mole) of methyl iodide added. Stirring and reflux were continued for 2½ hours and after cooling, 20 ml. of water was added. The solution was evaporated under reduced pressure leaving 28.8 Gm. (85%) of the crude product melting at 222–226°. The product was recrystallized from ethanol and melted at 229–231°.

3-Aza-1,5-diaminomethyl-6,6-dimethyl-bicyclo-(3.1.1)-heptane.—Lithium aluminum hydride, 8.4

[2] Prepared according to directions given by Rapoport and Payne (10).

[3] This and the following procedures are typical of those used for the preparation of compounds listed in Tables I and II.

Gm. of 95% purity (0.21 mole), was crushed and suspended in a mixture of 150 ml. of absolute ether and 50 ml. of absolute tetrahydrofuran contained in a 2-L., three-necked, round-bottom flask fitted with a dropping funnel, stirrer, condenser, and drying tube. The suspension was heated under reflux for 1 hour, cooled and 10.2 Gm. (0.05 mole) of 3-aza-1,5-dicyano-6, 6-dimethyl-2, 4-dioxo-bicyclo-(3.1.1)-heptane dissolved in 350 ml. of absolute tetrahydrofuran was added with stirring. After the addition was completed, stirring and reflux were continued for 9 hours. The reaction mixture was cooled and the complex decomposed by the cautious addition of 9 ml. of water, 9 ml. of 15% sodium hydroxide solution, and 27 ml. of water. The granular precipitate that had formed was filtered and washed with ether, the washings being added to the previous filtrate which was then dried with anhydrous sodium sulfate. The solvent was removed under reduced pressure and the residue of 3.4 Gm. distilled. The fraction boiling between 109–110°/0.16 mm. was collected and weighed 1.8 Gm. (20%).

The distilled material solidified at room temperature and attempts to prepare solid derivatives from it resulted in hygroscopic compounds which could not be purified.

3-Aza-1,5-diaminomethyl-6-ethyl-6-methyl-bicyclo-(3.1.1)-heptane Tripicrate.—Reduction of the bicyclo Guareschi imide derived from 2-butanone yielded 16% of the product. However, the material was too hygroscopic to have analyzed. A picrate derivative was readily formed but decomposed into picric acid and the triamine when recrystallization from conventional solvents was attempted. A stable tripicrate was prepared by dissolving 0.5 Gm. of the triamine in 1 ml. of absolute ethanol and slowly adding 22 ml. of a saturated solution of picric acid in absolute ethanol. The precipitated derivative was collected and purified by washing it with chloroform and decanting the solvent. This process was repeated six times using ethanol as the washing medium and finally the solid derivative was dried in a vacuum, m.p. 245–248° (decompn.).

SUMMARY

1. Five Guareschi imides were prepared by known procedures.

2. Selective reduction of the imide grouping of the Guareschi imides with lithium aluminum hydride uniformly resulted in ether-insoluble semisolids from which no pure product could be isolated or characterized.

3. Total reduction of the imide grouping and the nitrile groups of the Guareschi imides gave similarly poor results.

4. A series of four bicyclo Guareschi imides was prepared from the monocyclic Guareschi imides.

5. The four bicyclo Guareschi imides prepared readily underwent N-methylation.

6. Lithium aluminum hydride reduction of the four bicyclo Guareschi imides and their N-methyl analogs yielded the expected totally reduced products.

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(10) Rapoport, H., and Payne, G. G., *J. Org. Chem.*, **15**, 1093(1950).

ERRATUM

In the paper titled "Proposed Method of Assay for Diaphene" (1), the formula for calculating the per cent of Diaphene in paragraph three at page 45 should read:

$$\text{per cent Diaphene} = \frac{A \times 50 \times 100}{I \times V \times W} \times 100$$

(1) Soliman, S. A., and Harris, L. E., THIS JOURNAL, **52**, 43(1963).

Gm. of 95% purity (0.21 mole), was crushed and suspended in a mixture of 150 ml. of absolute ether and 50 ml. of absolute tetrahydrofuran contained in a 2-L., three-necked, round-bottom flask fitted with a dropping funnel, stirrer, condenser, and drying tube. The suspension was heated under reflux for 1 hour, cooled and 10.2 Gm. (0.05 mole) of 3-aza-1,5-dicyano-6, 6-dimethyl-2, 4-dioxo-bicyclo-(3.1.1)-heptane dissolved in 350 ml. of absolute tetrahydrofuran was added with stirring. After the addition was completed, stirring and reflux were continued for 9 hours. The reaction mixture was cooled and the complex decomposed by the cautious addition of 9 ml. of water, 9 ml. of 15% sodium hydroxide solution, and 27 ml. of water. The granular precipitate that had formed was filtered and washed with ether, the washings being added to the previous filtrate which was then dried with anhydrous sodium sulfate. The solvent was removed under reduced pressure and the residue of 3.4 Gm. distilled. The fraction boiling between 109–110°/0.16 mm. was collected and weighed 1.8 Gm. (20%).

The distilled material solidified at room temperature and attempts to prepare solid derivatives from it resulted in hygroscopic compounds which could not be purified.

3-Aza-1,5-diaminomethyl-6-ethyl-6-methyl-bicyclo-(3.1.1)-heptane Tripicrate.—Reduction of the bicyclo Guareschi imide derived from 2-butanone yielded 16% of the product. However, the material was too hygroscopic to have analyzed. A picrate derivative was readily formed but decomposed into picric acid and the triamine when recrystallization from conventional solvents was attempted. A stable tripicrate was prepared by dissolving 0.5 Gm. of the triamine in 1 ml. of absolute ethanol and slowly adding 22 ml. of a saturated solution of picric acid in absolute ethanol. The precipitated derivative was collected and purified by washing it with chloroform and decanting the solvent. This process was repeated six times using ethanol as the washing medium and finally the solid derivative was dried in a vacuum, m.p. 245–248° (decompn.).

SUMMARY

1. Five Guareschi imides were prepared by known procedures.

2. Selective reduction of the imide grouping of the Guareschi imides with lithium aluminum hydride uniformly resulted in ether-insoluble semisolids from which no pure product could be isolated or characterized.

3. Total reduction of the imide grouping and the nitrile groups of the Guareschi imides gave similarly poor results.

4. A series of four bicyclo Guareschi imides was prepared from the monocyclic Guareschi imides.

5. The four bicyclo Guareschi imides prepared readily underwent N-methylation.

6. Lithium aluminum hydride reduction of the four bicyclo Guareschi imides and their N-methyl analogs yielded the expected totally reduced products.

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Stability of Certified Dyes in Tablets I

Fading of FD&C Red No. 3 in Tablets as a Function of Concentration, Time, and Light Intensity

By MARTIN E. EVERHARD and FRANK W. GOODHART

The effect of concentration, time, and light intensity on the rate of fading of FD&C Red No. 3 in tablets has been examined. By modification of the Nolan and Foote equation relating the Kubelka-Munk function, θ, to dye concentration, a linear relation between the concentration of dye in the tablet and the θ calculated from the reflectance was found. A first-order rate equation for fading is given. This equation allows the fading which occurs under normal light intensity to be calculated from the fading which occurs at high light intensity.

One of the recurrent pharmaceutical problems is the instability of certified dyes in tablet formulations. The problem has historically been solved by selecting dyes which exhibit good stability in similar formulations. Within the last few years, several investigators have undertaken studies to determine the color stability by measuring the reflectance properties of the formulation.

Reflectance measurements of creams, powders, granulations, and coated tablets were made by McKeehan and Christian (1) using a modified Beckman DU spectrophotometer. This type of study was made quantitative by Lachman, *et al.* (2–5), who observed the changes in reflectance of tablets with time when exposed to visible radiation. In this latter case, the fading of the dyes was accelerated by using a high intensity light cabinet (6, 7). A plot of log (−log reflectance) *vs.* time demonstrated the fading rates of the dyes to be apparent first order.

It is necessary, however, to develop equations for relating dye fading as a function of time and light intensity so that more fundamental aspects of color degradation can be studied. Qualitative studies in our laboratory have shown that certain drug-dye incompatibilities alter fading and that, furthermore, tablet diluents, binders, lubricants, and processing methods may also do the same. Whether these factors are significant has not been determined. The dye concentration is also an important factor since apparent increased stability can sometimes be gained by increasing concentration. Therefore, we have conducted a series of experiments to determine the effect of dye concentration on the stability of FD&C Red No. 3[1] in tablets, and have discovered certain time-intensity relationships which are of significant importance to the research pharmacist.

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EXPERIMENTAL

Materials.—Lactose U.S.P., acacia U.S.P., magnesium stearate U.S.P., and FD&C Red No. 3.

Equipment.—Light sources were a bank of G.E. F4OCW fluorescent tubes, an incandescent lamp of 25 watts, and a light cabinet similar to that previously described (6, 7). The cabinet was equipped with a G.E. No. 6G1020-150 watts ballast and No. F40CW-3 fluorescent tubes. The tubes were 1.5 in. in diameter and 48 in. long, slant line cool white. They were G.E. rated at 3250 lumens per each 40 watt tube.

The temperature of the tablets was about 22 to 24°, except for the tablets stored in the light cabinet. The temperature of the tablets in the light cabinet was 2 to 3° higher than room temperature.

A Bausch and Lomb Spectronic 505 spectrophotometer with a reflectance attachment was used to obtain tablet reflectances. Magnesium carbonate was used as a white standard. A Gossen Tri-Lux foot-candle meter employing a microgalvanometer connected to the photoelectric cell by a 3-ft. extension cord was utilized to determine incident flux.

Preparation of Tablets.—Tablets were prepared according to the following three formulas

	L	M	N
Lactose U.S.P., %	93.940	93.970	93.985
Acacia U.S.P., %	5.000	5.000	5.000
Magnesium stearate U.S.P., %	1.000	1.000	1.000
FD&C Red No. 3, %	0.060	0.030	0.015
Distilled water, ml.	50	50	50

Lactose and acacia were mixed together and pulverized. The dye, dissolved in 50 ml. of distilled water, was poured onto the powder mixture while mixing. The wet powder was dried at 75° F. and 40% relative humidity for 16 hours, and then in an oven at 100° F. for 2 hours. The dyed powder was pulverized and the magnesium stearate added and

[1] Disodium salt of 9-o-carboxyphenyl-6-hydroxy-2,4,5,7-tetraiodo-3-isoxanthone (erythrosine).

blended with the powder. Uniformly colored, flat-faced pink slugs were produced on the Stokes F-4 tablet press using 1-in. punches and die. The tablets were run at uniform weights and pressures in order to produce tablets having consistent surface properties. The free moisture content of the tablets was about 1%.

Preparation of Tablet Samples.—Tablets were glued on large, black rubber stoppers.[2] The stoppers were marked so that the geometry could be reproduced when returning the sample to the reflectance sample holder.

Storage of Tablets.—Prepared tablet samples were exposed under the following conditions: (*a*) in the light stability cabinet at a flux of 655 foot-candles, (*b*) under a bank of fluorescent lights at 80 foot-candles, (*c*) under fluorescent lights and amber glass (0.120 in.) at 11 foot-candles, and (*d*) under incandescent light at 50 foot-candles.

DISCUSSION AND RESULTS

Previous workers have plotted the logarithm of the function log $1/R$ *vs.* time, where R is the fraction of the light reflected at a specified wavelength. They interpreted the results in terms of apparent first-order reactions (3). Although they did not explicitly state that log $1/R$ is a linear function of dye concentration, just as log transmission is a linear function of concentration in transmission work, this is inferred because they interpret their results in kinetic terms (8). However, when this assumption was tested at different dye concentrations, a new "kinetic" constant, k, was found for the rate equation at each concentration, although the results for any one concentration were linear and reproducible. Since k is different for each concentration, the reaction is either not first order or an incorrect rate equation was used. In the present work, doubling the dye concentration resulted in a 20% change in k when plots of log R *vs.* time were made. Similar results were obtained by using R *vs.* time, that is, zero-order plots. Since the reflectance value is not proportional to concentration, an expression relating these two quantities is needed.

From theoretical consideration Kubelka and Munk (9, 10) derived a series of differential equations describing the behavior of light when it strikes a diffusing surface. These equations can be combined and integrated to give one simple equation if certain assumptions are met

$$\theta = (1 - R)^2/2R \qquad \text{(Eq. 1)}$$

Where θ is the ratio of absorbed light to scattered light at a given wavelength. The most important assumptions are that the material is homogeneous, that it has sufficient thickness to be opaque, and that the reflectance is diffuse. These assumptions are easily met by using uniformly dyed tablets with a dull surface.

Nolan (11) and Foote (12), in working with the Kubelka-Munk function, found that if the scattering coefficient is assumed to be very small, zero, or constant, θ is proportional to concentration. The scattering coefficient is the fraction of incident light lost due to scattering. If this is assumed, then

$$\theta_d = AC \qquad \text{(Eq. 2)}$$

where θ_d is the Kubelka-Munk function for the dye, A is a constant of proportionality, and C is the concentration of dye in weight per cent. The observed reflectance, however, is due to the dye and the inert, background materials in the formulation. Nolan showed that the Kubelka-Munk function of a mixture is the sum of the individual Kubelka-Munk functions. Therefore

$$\theta_t = \theta_d + \theta_b \qquad \text{(Eq. 3)}$$

where θ_t is the Kubelka-Munk function of the tablet and θ_b of the background. Substituting Eqs. 1 and 2 into 3 gives

$$\theta_t = (1 - R_t)^2/2\,R_t = AC + \theta_b \qquad \text{(Eq. 4)}$$

where R_t is the measured reflectance of the tablet at the minimum in the reflectance-wavelength curve, 543 mμ.

Since the concentration of inert material, mostly lactose, is essentially constant, θ_b is a constant. Therefore, a plot of θ_t *vs.* C, Eq. 4, should be a straight line. By using the smoothed values of θ_t at zero time from Fig. 1, a straight line is obtained. The value of A is 8.2 and θ_o is 0.146, within 2%. This shows that θ_t is linearly related to the concentration and suitable for use in kinetic plots.

Previous attempts to correlate the fading of tablets at different intensities of the incident light have not been satisfactory. Each intensity was considered a separate case and different kinetic constants were calculated for each intensity (3, 4). However, since fading is proportional to the product of time, t, and intensity, I (13), plots of θ_t *vs.* the product of time and intensity were made. The plots are shown in Fig. 1. The consistency of the k value at the three different concentrations shows that the decomposition of the dye is indeed first order. By using the product of time and intensity, each intensity condition was found to lie on the same straight line for any given concentration.

The general first-order rate equation is

$$\ln \theta_t = -ktI + \ln \theta_t' \qquad \text{(Eq. 5)}$$

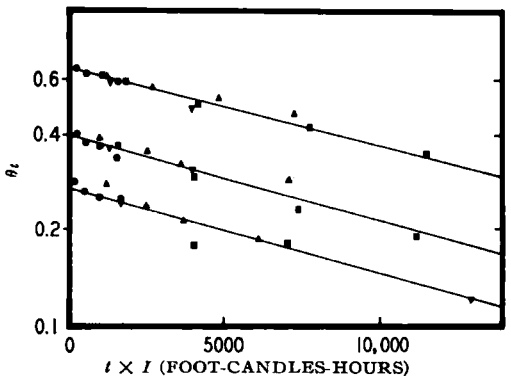

Fig. 1.—Plots of θ_t *vs.* the product of time and intensity. ●, 11 foot-candles; Δ, 50 foot-candles; □, 80 foot-candles; ▽, 655 foot-candles. Top line, 0.06% dye; middle line, 0.03% dye; bottom line, 0.015% dye.

[2] Our thanks to Mr. D. Dickcius for devising this easy method of mounting the samples and for taking many of the reflectance measurements.

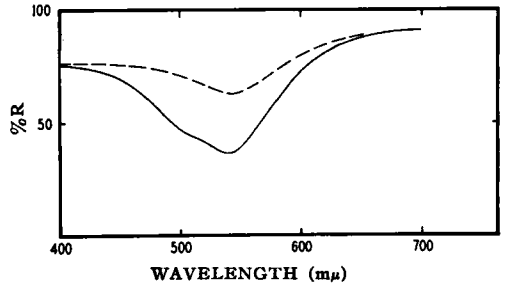

Fig. 2.—Reflectance curves for 0.06% dye. Solid line, initial; dashed line, after 140 hours at 80 foot-candles.

where θ_t is the θ calculated at time t, θ_t' is the θ_t at $t = 0$, and the other symbols have the meanings explained above. The rate constant was calculated to be $6.1 \pm 0.3 \times 10^{-5}$ hr.$^{-1}$ foot-candles^{-1}.

Figure 2 shows the 543 mμ absorbance peak and the shape of the reflectance curve before and after fading. Since the rate of fading is the same for incandescent and fluorescent illumination, as well as for samples protected by amber glass, the decomposition must be due largely to absorption at wavelengths greater than 500 mμ.

The times at which objectionable fading under high light intensity occurred for this dye, in the opinion of the authors, at all three concentrations are shown in Table I. From these time-intensity values, the time for objectionable fading at lower intensities was calculated using Eq. 5. The advantage of using high intensity lighting conditions is obvious from this table.

TABLE I.—TIME REQUIRED FOR OBJECTIONABLE FADING AT LOW LIGHT INTENSITIES AS CALCULATED FROM ACCELERATED LIGHT CONDITIONS

Concentration of Dye, % w/w	Hours at 540 f.-c.	Calcd. Hours at 50 f.-c.	Calcd. Hours at 10 f.-c.
0.060	17	180	920
0.030	7	75	380
0.015	2	20	110

Examination of the cross section of the tablets showed that fading proceeds to a given depth into the tablet and a fairly sharp demarcation can be seen between the white faded portion and the unchanged dye. The fading occurs first at the surface and gradually progresses into the tablet. When the tablet is viewed from above, the pink color seems to fade uniformly, indicating that the lower layers of dye are responsible for some of the surface color.

When the fading had penetrated to about 0.3 mm. into the tablet, the tablet surface appeared white and no further increase in the thickness of the white layer occurred.

The faded top layer did not interfere with the measurements of the reflectance until about 15,000 foot-candle-hours. After that, the fading rate slowed, probably because the upper layers of faded dye were thick enough to offer some protection to the lower, visible layers. The lower fading rate was obtained only in the light cabinet and is not shown in Fig. 1.

This work will be continued by measuring rate constants for other dyes using the equations developed above. It is hoped that when enough experience is obtained, it will be possible to predict the rate of fading for various combinations of dyes in the same tablet or tablets of different composition. By use of the present data, it is now possible to predict the amount of fading of FD&C Red No. 3 in the formulation studied at any specified time under any light intensity.

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Drying as a Unit Operation in the Pharmaceutical Industry I

Drying of Tablet Granulations in Fluidized Beds

By M. W. SCOTT, H. A. LIEBERMAN, A. S. RANKELL, F. S. CHOW, and G. W. JOHNSTON

The results of drying tests in a fluidized bed and a tray dryer are reported for typical tablet granulations. The data are analyzed to give estimates of total drying times, rates of drying, and overall heat transfer coefficients in each unit. It is concluded that fluidized bed drying of tablet granulations is at least 15 times faster than tray drying procedures. Additional factors, such as capacities per unit floor space, operating costs and thermal efficiencies are reviewed and illustrate other advantages of the fluidization technique.

DRYING procedures, like other important unit operations in pharmacy, such as size reduction, solids blending and liquid agitation, have received little attention in pharmaceutical literature. Within recent years, however, growing interest in these pharmaceutical engineering areas has become apparent. This report on fluidized bed drying is one in a series by the authors in this new investigative field of pharmacy.

Fluidization operations, although relatively unexplored in pharmacy, have been firmly established on a broad scale in other industries. Textbooks and other extensive references on fluidization have been published during the past 20 years (1–5). However, relatively few reports have appeared on the applications of fluidized beds to materials of pharmaceutical interest (6–10).

Fluidized bed drying of coal, sand, plastics, and numerous other materials have been discussed in technical literature (11–14). These studies show that high heat transfer, mass transfer and drying rates are obtainable in fluidization systems. As a result of turbulence and excellent interphase contact, uniform bed temperatures are readily achieved. Product temperatures also are controllable over narrow limits within the fluidized bed. These reported advantages would appear to have particular importance in processing pharmaceuticals. It is surprising to note, therefore, that no studies on fluidized bed drying have appeared in pharmaceutical literature.

The object of the work conducted in the present report was to investigate the applications, advantages, and limitations of fluidization techniques in drying pharmaceutical tablet granulations. A representative, commercially available, fluidized bed dryer was used for these studies. Comparative data were obtained from drying tests with a conventional tray dryer.

EXPERIMENTAL

Materials.—Two tablet granulations were used in this study. The first was a lactose placebo made with a solution of methylcellulose as the binding agent. This granulation was prepared also with added color (FD&C No. 1 Red). The second test granulation was an antacid consisting primarily of magnesium trisilicate with syrup as the binding agent. These granulations were prepared by conventional techniques and screened through a standard No. 4 sieve before drying.

Equipment.—The fluidized bed dryer used in this work was the Glatt dryer, laboratory model TR-5.[1] Figure 1 gives an illustration of the equipment, which consists of a fan, a drying chamber fitted with a wire mesh support for the granulation, a bag dust collector, an air filter, and electrical heating elements. Controls are provided for adjustment of air flow (by damper settings) and inlet air temperatures.

The instrument operates by forcing, by induced draft, heated air through the drying chamber at

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[1] The authors express their appreciation to the Chemical and Pharmaceutical Industry Co., Inc., New York, N. Y., for loan of the Glatt dryer, manufactured by Werner Glatt, Inc., West Germany.

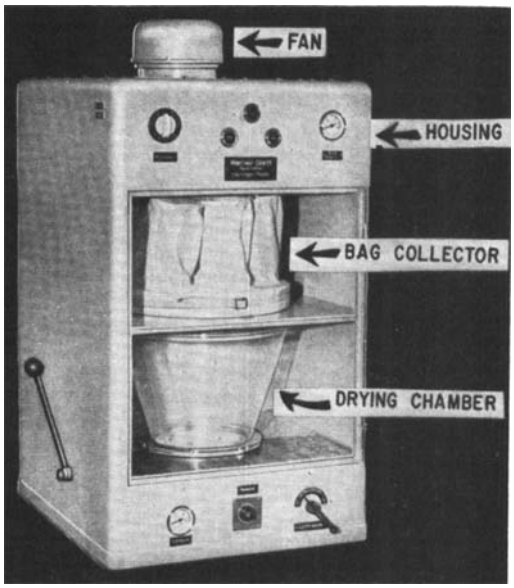

Fig. 1.—Glatt TR-5 dryer.

velocities which are sufficient to fluidize the granulation charged in the chamber. The air escapes through the bag collectors and is vented through an exit port. Granulation is quickly charged and discharged from the drying chamber by dumping. The Glatt dryer was modified for this study by attaching auxiliary thermometers within the drying chamber and immediately outside the dust collector bag.

The tray dryer used in these experiments was a Stokes dryer, model 38A, fitted with 16 shelves and automatic thermostat controls. Damper positions for air recirculation were set in accordance with the manufacturer's recommended operating directions. Air temperatures were measured with auxiliary thermometers installed within the inlet and outlet air ducts. Bed temperatures were obtained from thermometers inserted into the granulation on the bottom, middle, and top trays.

Drying Methods.—All experiments were performed in an air conditioned, temperature-controlled area. Readings of relative humidity were taken throughout each run and found to be relatively constant at 40 to 45%.

Equilibrium inlet air conditions were established at the start of each fluidized bed run. All experiments were conducted with a 5-Kg. charge of wet granulation except where the load was the variable. In these latter experiments, loads of 1, 3, 5, and 7 Kg. were employed. Inlet air temperatures (3 levels) and air velocities (2 levels) were the other experimental variables studied.

Tray dryer experiments were run, using 40 Kg. of placebo granulation (full dryer capacity) as the charge. This was equally distributed by weight over each tray to give a uniform bed thickness of approximately one inch. Inlet air temperatures were varied (5 levels) in these experiments. In one drying experiment the same colored placebo test granulation was used that had been studied by the fluidized bed technique.

Product Evaluation Techniques.—Aliquots of granulation were obtained by combining at least 10 random spot samples from representative locations within each drying unit. Samples and instrument measurements were taken at 5- or 10-minute intervals in fluidized bed experiments and at hourly intervals in the tray dryer studies. Moisture analyses were performed on each sample, using a Cenco moisture balance set at 90 volts for 6 minutes. Replicate determinations established the levels of reproducibility at ±0.2%. Screen analyses were performed on 100-Gm. coned and quartered aliquots of the total dried product collected in each run. Portions of the dry granulations then were mixed with lubricant and compressed into tablets. Compression characteristics were noted for each test granulation. Tablets made from the colored placebo granulations were inspected further for mottling and overall color uniformity.

DISCUSSION OF RESULTS

Engineering Analysis of Fluidized Bed Drying.—Figure 2 presents typical data for drying the placebo and antacid granulations in the fluidized bed. Moisture contents are expressed here on a wet basis in terms of the weight loss on drying or L.O.D. values. This method of expressing moisture has been employed in the pharmaceutical industry and is defined by Eq. 1

$$\% \text{ L.O.D.} = (100)\ \frac{x}{y + x} \qquad \text{(Eq. 1)}$$

where x = weight of water, y = weight of dry solid.

L.O.D. values were obtained directly by analysis with the Cenco moisture balance. Equilibrium L.O.D. values for each drying run were obtained by inspection of the plots of L.O.D. *vs.* time. In Fig. 2, for example, the equilibrium L.O.D. for the dried placebo and antacid granulation were found to be 0.9 and 3.0%, respectively. Over the range of inlet air conditions studied, equilibrium values were found to fall in the range of 1.1 ± 0.2% for all

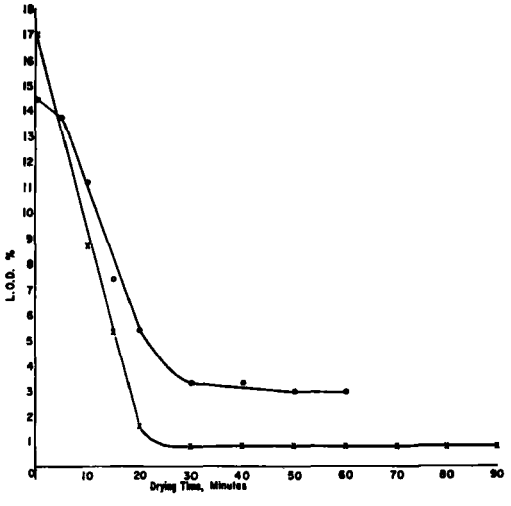

Fig. 2.—Loss on drying *versus* time for placebo and antacid granulation in fluidized beds. ×— Placebo, run 2; O— antacid, run 13.

placebo runs and 3.2 ± 0.2% for all antacid tests. These moisture levels were consistent with those desired for good compression characteristics of each granulation.

The total processing time required to dry the test granulations to their equilibrium L.O.D. values also were obtained from drying curves of the type illustrated in Fig. 2. The drying times for runs 2 and 13 were 24 and 30 minutes, respectively. For other runs in the fluidized bed, total drying times ranged from approximately 10 to 62 minutes. Table I reviews the conditions employed in these experiments and summarizes the data for fluidized bed drying. The rapidity of the fluidized bed drying technique is demonstrated by drying time results shown in Table I.

Inspection of drying data on the basis of L.O.D. values serves as a convenient starting point for comparison of drying operations. For a more detailed analysis, however, it is generally preferable to express moisture contents on a dry basis, *i.e.*, % M.C., defined by Eq. 2

$$\% \text{ M.C.} = (100)\,\frac{x}{y} \qquad \text{(Eq. 2)}$$

where x = weight of water, y = weight of dry solid.

The conversion of % L.O.D. to % M.C. is expressed by Eq. 3

$$\% \text{ M.C.} = \frac{\% \text{ L.O.D.}}{100\% - \% \text{ L.O.D.}} \qquad \text{(Eq. 3)}$$

Figure 3 gives the moisture content (% M.C.) histories for runs 2 and 13 and is representative of the other fluidized bed experiments. Estimates of drying rates were obtained from these curves by graphic determinations of slope values. Next, rates of drying were plotted as a function of drying time, as illustrated in Fig. 4. A semilogarithmic graph was employed here for better visualization of the slower drying rates. In passing, it should be noted that L.O.D. values as such cannot be used for calculation of drying rates. The basis for these values (wet basis) changes continuously as drying proceeds, thereby introducing significant errors into the rate determinations.

Data presented in Fig. 4 for run 13 (antacid) exhibit the typical rate patterns observed in drying studies (15, 16). Three major drying rate periods are apparent. The first is a period of initial rate adjustment which lasts approximately 5 minutes. This is followed by a constant drying rate period of about 15 minutes duration. Continued drying then occurs at falling rates. This last interval is referred to as the falling rate period. Similar constant and falling rate periods are seen with the placebo granulation (run 2). In this case, however, experimental demonstration of an initial rate adjustment period was not obtained.

Rate data were also examined as a function of granulation moisture content, as illustrated in Fig. 5. The various rate periods and the critical moisture content values (defined as the moisture content which terminates the constant rate period) are readily identified. For experiments 2 and 13,

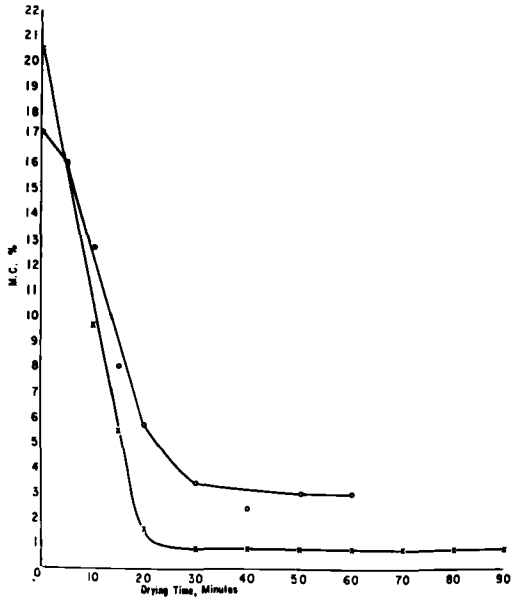

Fig. 3.—Moisture content *versus* time for placebo and antacid granulation in fluidized beds. ×— Placebo, run 2; O— antacid, run 13.

TABLE I.—DATA FOR FLUIDIZED BED DRYING

Run[a]	Bed Load on Wet Basis, Kg.	Inlet Air, °F.	Damper Setting	Time to Reach Equilibrium L.O.D.,[b] min.	Duration of Constant Rate Period, min.	Critical Moisture Content, Per Cent	Rate of Drying[c] Kg. (H_2O)/min./ Kg. (Dry Product)
1P	5	125	2	35	20	2.0	8.2×10^{-3}
2P	5	128	2	24	20	1.7	9.4×10^{-3}
3P	5	126	6	25	15	3.0	12×10^{-3}
4P	5	108	2	62	55	2.4	3.4×10^{-3}
5P	5	113	6	57	15[d]	4.0	10×10^{-3}
6P	5	139	2	25	8	7.6	14×10^{-3}
7P	5	150	6	16	13	1.4	13×10^{-3}
8P	7	129	6	38	20	3.1	7.6×10^{-3}
9P	3	129	6	18	10	2.7	16×10^{-3}
10P	1	131	6	10	5	2.0	31×10^{-3}
11A	5	115	2	60	35[d]	5.5	3.9×10^{-3}
12A	5	129	2	60	30	6.8	4.5×10^{-3}
13A	5	145	2	30	15[d]	4.9	7.4×10^{-3}
14A	5	147	2	40	22[d]	4.8	6.2×10^{-3}

[a] P = Placebo; A = Antacid. [b] Equilibrium L.O.D. values for all runs were found to be 1.1 ± 0.2% for the placebo granulation and 3.2 ± 0.2% for the antacid granulation. [c] Constant rate period. [d] Duration of initial rate adjustment period was less than 10 min. In all other runs duration was less than 5 min.

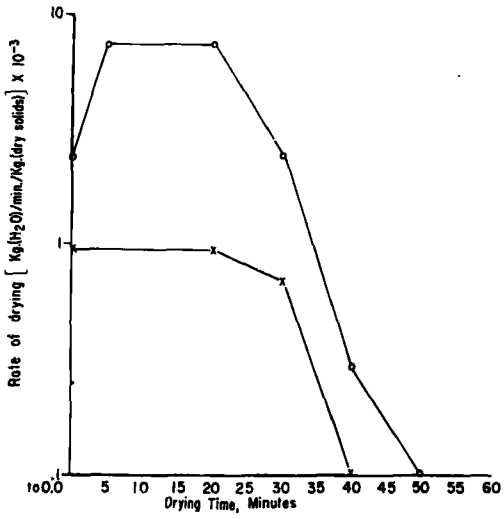

Fig. 4.—Rate of drying *versus* drying time for placebo and antacid granulation in fluidized beds. ×— Placebo, run 2; O— antacid, run 13.

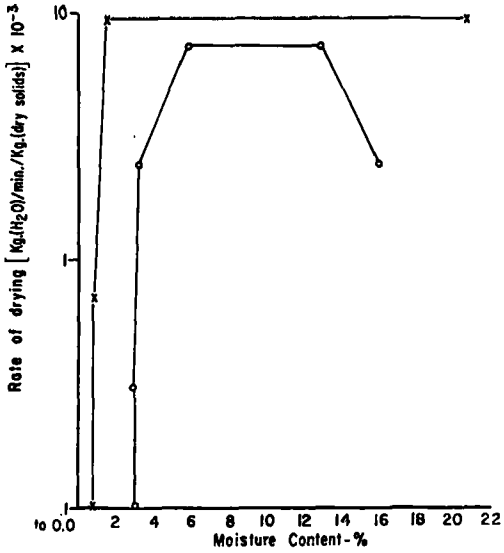

Fig. 5.—Rate of drying *versus* moisture content for placebo and antacid granulation in fluidized beds. ×— Placebo, run 2; O— antacid, run 13.

critical moisture contents were estimated at 1.7 and 4.9%, respectively. Critical moisture content values for other drying runs are listed in Table I.[2]

Theoretical mechanisms for moisture movement during each rate-of-drying period have been reviewed elsewhere (15, 16). In general, constant drying rates occur during the period when the surface of the solid is completely wet with a thin film of unbound moisture. Water that is evaporated from the surface during this interval is immediately replaced by moisture diffusing out from within the solid. Constant rates of drying are maintained so long as the rate of internal moisture diffusion equals the rate of surface evaporation. As drying proceeds, a condition eventually is reached at which the surface film of moisture can no longer be replenished completely by internal moisture diffusion. At this point (critical moisture content), the surface of the solid becomes partially dry and the rate of drying decreases. This initiates the period of falling drying rate. During the falling rate period, mass transfer (diffusion) effects within the solid control the course of drying. During the constant rate periods, external factors which influence heat transfer to the surface and mass transfer from the surface govern the operation.

Each drying period may be analyzed separately to simplify mathematical treatments and correlations of the drying variables. If the bulk of the moisture is evaporated during constant rate periods, analysis of this period alone will be sufficient for definition of the drying operation. For the experiments reported in this study, inspection of the curves of the rate of drying *vs.* moisture content, as illustrated in Fig. 5, indicated that evaporation occurred predominantly under constant rate conditions. Major emphasis, therefore, was placed on analysis of this drying period.

Data for the constant rate periods of drying are given in Table I for all fluidization runs. Rate of drying is defined by Eq. 4.

$$R = \frac{\text{M.C.}_s - \text{M.C.}_e}{\theta} \qquad \text{(Eq. 4)}$$

where R = rate of drying—Kg. (H_2O)/min./Kg. (dry solid); M.C.$_e$ = moisture content at end of constant rate period (critical moisture content)—Kg. (H_2O)/Kg. (dry solid); M.C.$_s$ = moisture content at the start of constant rate period—Kg. (H_2O)/Kg. (dry solid); θ = duration of constant rate period (minutes). All values in Eq. 4 are obtainable directly from the plots of rates of drying *vs.* drying time (Fig. 4) and rates of drying *vs.* moisture content (Fig. 5).

Constant rate values for fluidized bed drying of the placebo granulation using 5-Kg. loads ranged between 3.4 and 14 × 10^{-3} Kg. of water evaporated per minute per Kg. of dry product, as shown in Table I. Drying rates for the antacid were of the same order of magnitude. Comparison of rates of drying at approximately equal inlet air temperatures shows, however, that the placebo generally was easier to dry than the antacid granulation. This conclusion is supported further by the data for drying times listed in Table I.

As expected, increasing the bed load in the fluidization runs was found to have an adverse effect on the drying rate. This is shown in Fig. 6. The derivation of the semilogarithmic correlation obtained here is not fully understood at present. It is believed to result in part from the influence of the conical geometry of the Glatt drying chamber on the height of the bed at different charge weights.

Although drying rates decreased with increased loads, overall processing rates were shown to be highest when 5 and 7 Kg. of granulation was used as charge. In the present experiments, loading and emptying of the fluidized bed was accomplished in less than 2 minutes. This handling time was added to the drying time data listed in Table I to give

[2] Critical moisture contents are not constants, but vary from material to material and with the drying conditions employed. In general, the values are expected to be higher at increasing drying rates (15). No correlations in this direction could be established, however, in the present study.

estimates of the total cycle times. Processing rates per unit weight of product (throughput) were obtained from these values and found to reach a maximum with 5 Kg. of charge. This is in line with the manufacturer's statement of optimum load for the model TR-5 dryer.

Figure 7 shows the relationship between the duration of the constant rate period and bed load. Extrapolation of the data to 0 Kg. was used to give an estimate of the duration of the initial rate adjustment period, *i.e.*, the time when constant rate begins. An intercept value of 2.5 minutes was obtained under the inlet air conditions employed for these load experiments. The duration of this period was expected (15) and observed to change with varying inlet air temperatures.

During constant rate periods, rates of drying can be based on heat transfer relationships as shown in Eq. 5

$$R = \frac{h_t(\Delta T)}{DL\lambda} = K(\Delta T) \qquad \text{(Eq. 5)}$$

where R = rate of drying—Kg. (H_2O)/min./Kg. (dry solid); D = density of dry solid—Kg./meter^3; L = thicknes of drying solid—meter; λ = latent heat of vaporizaton—B.t.u./Kg.; h_t = total heat transfer coefficient—B.t.u./min./meter^2/° F.; K = overall transfer coefficient—Kg. (H_2O)/min./Kg. dry solid/° F; ΔT = temperature gradient between drying air and product—° F. Plots of rates of drying *vs.* ΔT are expected to give linear relationships which pass through the origin as indicated by Eq. 5. The slope of the line is numerically equal to K, the overall transfer coefficient.

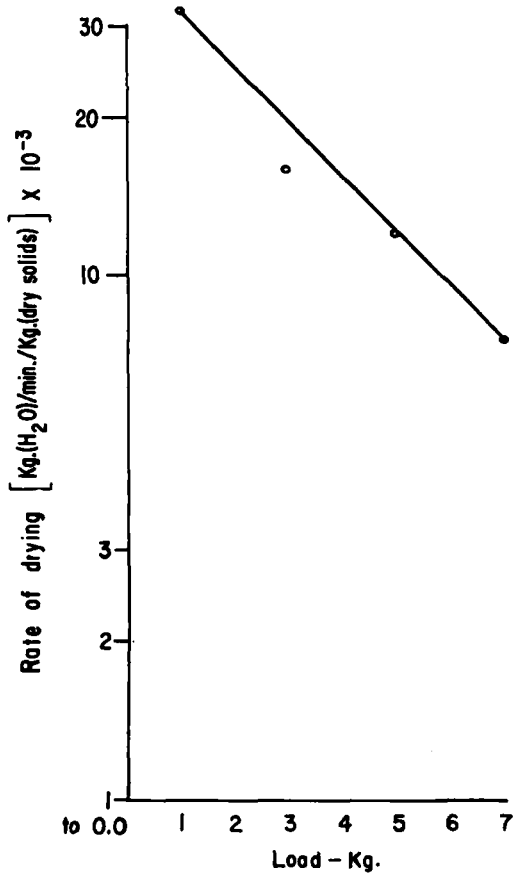

Fig. 6.—Plot of rate of fluidized bed drying *versus* bed load.

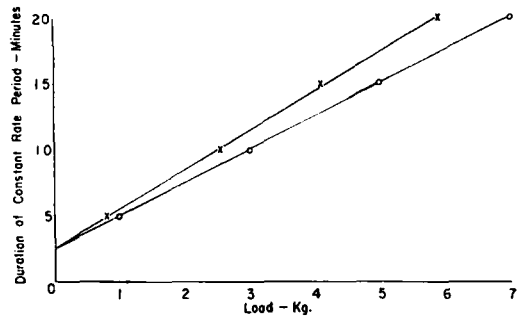

Fig. 7.—Plot of duration of constant drying rate *versus* bed load. X— Bed load expressed on dry basis; O— bed load expressed on wet basis.

Rate data for fluidized bed drying under two different inlet air velocity settings are plotted in Fig. 8. The temperature gradients used here were calculated from averaged inlet air temperatures and bed temperatures which existed during the constant rate period of drying. The linear relationship in Fig. 8 is considered satisfactory, particularly in view of the averaging procedures used in obtaining ΔT values. Results obtained for the two air velocity settings appear to follow identical relationships. This suggests that the transfer coefficient, K, is independent of air velocity settings over the range studied. Therefore, little or no increase in drying rates can be expected at the higher setting, setting 6.

The numerical value of the overall transfer coefficient obtained from the slope of the line in Fig. 8 is 2.8 × 10^{-4} Kg. of water evaporated per minute per Kg. of dry solid per ° F. This value does not compare favorably with published transfer coefficients for fluidized bed drying when all are expressed in common units (17, 18). Undoubtedly, the low Glatt value arises because the bed did not fluidize completely during all of the drying cycle. Efficient fluidization of the granulation occurred only after considerable drying had taken place. This lack of complete fluidization throughout the

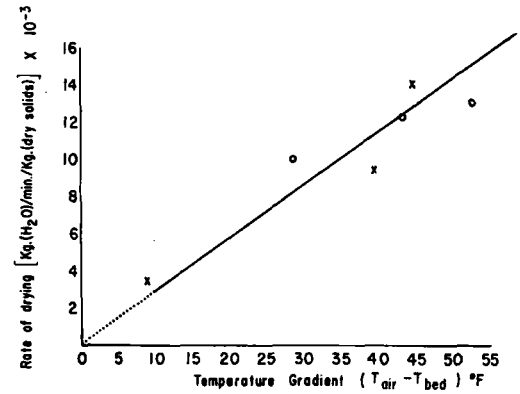

Fig. 8.—Rate of fluidized bed drying *versus* thermal gradient. O— Fan setting 6; X— fan setting 2.

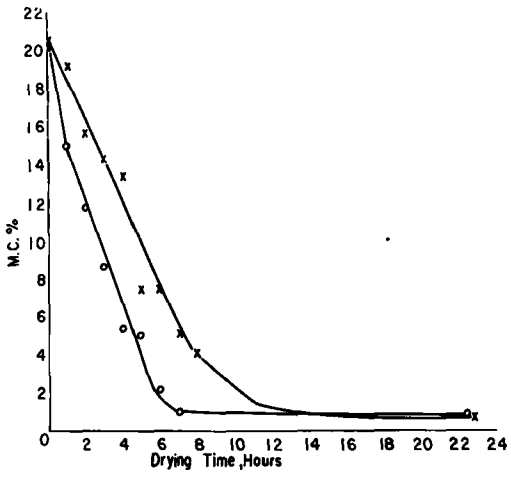

Fig. 9.—Moisture content *versus* drying time for two placebo granulations in tray dryer. X— Placebo, run 15; O— placebo, run 16.

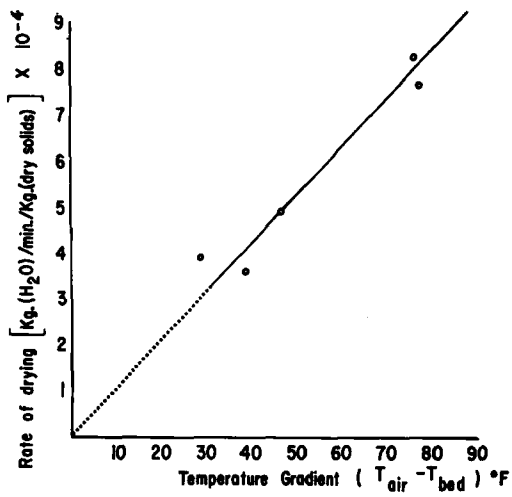

Fig. 10.—Rate of drying *versus* thermal gradient in tray dryer.

entire drying cycle represents an area for needed improvement in the Glatt equipment.

Comparison of Tray Drying and Fluidized Bed Drying.—Data collected for drying the placebo granulation in tray dryers was subjected to the same analysis techniques used above. Figure 9 illustrates moisture content histories for two typical runs. Pertinent operating and performance data for all tray experiments are summarized in Table II. Results of the present study show that drying times ranging from about 330 to 720 minutes are required in tray drying operations. These values are significantly higher (by a factor of about 20) than those observed in fluidized bed drying. Direct comparisons of the two operations on the basis of drying times alone are not completely valid, however, because different loads are handled in each unit.

Meaningful comparisons of tray and fluidized bed drying can be obtained using rate data which have been expressed on a common weight basis as listed in Tables I and II. Rates of tray drying during the constant rate period were found to be in the range of 3.6–8.2 $\times 10^{-4}$ Kg. of water evaporated per minute per Kg. of dry solid (Table II). These rates are approximately 15 times lower than those obtained in the fluidized bed drying and again indicate the comparative rapidity of the fluidization technique.

Rates of tray drying noted above are equal to 0.02–0.05 when expressed in units of lb. of water evaporated per hour per lb. of dry solid. These constant rate values fall toward the lower side of the range (0.1–2.0 lb. per hour per lb.) previously reported for tray drying (19). Overall rates of tray drying have been reported also (15), and fall between 0.03–0.30 lb. of water evaporated per hour per ft.^{2} of tray surface. When calculated on this basis, the present data indicate a rate of approximately 0.02 lb. per hour per ft.^{2} which is slightly lower than expected. These results suggest, therefore, that areas for improved performance also may exist with the particular tray drying unit used here.

Figure 10 presents the plot of rate of drying *vs.* temperature gradient for the tray dryer studies. The agreement of the experimental data with the relationship expressed by Eq. 5 is satisfactory. The overall transfer coefficient determined from the slope of the line in Fig. 10 is equal to 1×10^{-5} Kg. of water evaporated per minute per Kg. dry solid per ° F. This value has the correct order of magnitude. It is lower, however, by at least a factor of 2 to 5 than the values previously reported in the literature for transfer coefficients (15).

It will be recognized that K values are independent of temperature gradient and weight of feed charge. Therefore, these coefficients also are useful indices for comparative evaluation of different drying procedures. Comparison of the transfer coefficients for the two drying operations studied in the present report shows that heat transfer is at least 28 times more effective in the fluidized bed than in the tray dryer. This is fully in accord with theoretical considerations and results from the very favorable interphase contact obtainable in fluidized beds. Relative thermal efficiencies between fluidized bed and tray drying pointed to other advantages of the fluidization technique in this study.

TABLE II.—ANALYSIS OF DATA FOR TRAY DRYING OF PLACEBO GRANULATION[a]

Run	Average Inlet Air Temperature, ° F.	Drying Time to Reach Equilibrium L.O.D., min.	Duration of Constant Rate Period, min.	Critical Moisture Content, Per Cent	Rate of Drying During Constant Rate Period Kg. (H_2O)/min./Kg. Dry Solid
15	131	720	420	5.3	3.6×10^{-4}
16	140	420	270[c]	3.3	4.5×10^{-4}
17	160	480	240[c]	3.2	4.9×10^{-4}
18	177	430	180	6.8	7.6×10^{-4}
19	191	330	216	3.2	8.2×10^{-4}

[a] Data for 40 Kg. charge to dryer. [b] Equilibrium L.O.D. values for all runs were found equal to 1.1 ± 0.2%. [c] Constant rate period began after 60-minute interval initial of rate adjustment.

Thermal efficiency was defined as the ratio of the minimum thermal energy theoretically required to dry the solid to the actual thermal energy used in the drying unit.[3] For the present experiments, a 2–6 fold advantage in thermal efficiency was determined for the fluidized bed operation. This can be related directly to potential fuel cost savings obtainable with the fluid bed.

Other Considerations.—Several additional advantages of fluidization techniques which have direct importance in the drying of tablet granulations were apparent in this study. Firstly, fluidized beds are turbulent systems which give rise to good mixing effects. As a consequence, uniform bed temperatures (product temperatures) are readily achieved and easily controlled. With a properly designed unit, local areas of overheating can be completely eliminated. In tray drying procedures, however, nonuniform tray and product temperatures are the rule rather than the exception. For example, in the present study it was observed that tray temperatures varied as much as ±7° from location to location within the dryer. The close control of product temperature in fluidized bed drying suggests that the operation will be particularly suited for processing heat-sensitive materials and granulations which tend to case harden.

Tablets made from granulations dried in the fluidized bed compared favorably in all respects to those made from tray dried granulations. Visual inspection of tablets made from the colored granulations showed further that improved color uniformity is obtained with the fluidized bed granulation.

Agitation and product turnover are inherent in fluidization. As a consequence, opportunities appeared to exist for blending lubricants and other materials with the dry granulation within the fluidized bed itself. The use of the fluidized bed for the blending of solids has been reported previously (20, 21), and this aspect was not explored further in the present study.

For tray drying procedures, it is generally agreed that the labor involved in loading and unloading represents about one-third of the total operating costs (15). About one man-hour was required for these steps in the present experiments. In comparison charging and emptying the fluidized bed dryer was completed in less than two minutes. On this basis, therefore, fluidized bed drying offers significant opportunities for reductions in labor costs.

Caking of the granulation was observed at the start of several of the fluidization runs. In one experiment (not reported in Table I), severe caking was noted when using a slightly overwet granulation. This decreased drying rates significantly and led to the development of many oversized granules. The results of this single experiment suggest, therefore, that tacky granulations may pose special difficulties in fluidized bed drying. Friable and dusty granulations also may be troublesome because of their tendency to clog the bag collectors.

In the majority of the fluidization experiments reported here, only small amounts of oversize granules (larger than 10 mesh) were present in the final dried product. The bulk of the granulation was suitably sized for compression without additional grinding operations. The elimination of this post-drying grinding step represents an important advantage of the fluidized bed technique.

The present experiments indicate that fluidized drying of tablet granulations may require special control of drying times. Turbulence in the fluidized bed was found to give varying degrees of particle attrition. These attrition effects became particularly significant when fluidization was continued for extended time intervals with dry granulations. The degree of attrition also was found to increase at the higher air velocity, damper setting 6. For example, in run 1 at damper setting 2, the final dry granulation contained 9.3% fines (less than 60 mesh) with 15% of the granulation larger than 10 mesh. At damper setting 6 (run 3), no particles larger than 10 mesh were present while fines increased to 15%. Under usual operating conditions, however, good reproducibility of granulation size distribution was readily obtainable.

A Glatt fluidized bed dryer of about 30 Kg. wet load capacity occupies slightly less floor space than the 40 Kg. tray dryer. It is reasonable to assume that with proper scale-up, drying times in the larger fluidized bed can be maintained at one-half hour. Production rates per unit floor space will be expected, therefore, to be at least 15 times higher than those obtainable by tray dryers.

CONCLUSIONS

The results of this study show that the fluidized bed dryer offers significant advantages over the conventional tray dryer commonly used in the pharmaceutical industry. These advantages are not limited to the particular commercial unit tested in this report but rather arise from the inherent qualities and characteristics of the fluidization process itself. These advantages include:

1. Increased rates of drying and product throughput, accompanied by significant improvements in thermal efficiency.
2. Increased drying capacities per unit floor space.
3. Increased ability to control product tem-

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[3] Thermal efficiency $= (100)\dfrac{E_T}{E_A}$ (Eq. 6)

E_T = minimum energy input theoretically required to dry the solid in B.t.u./min., while E_A = actual energy input used by the unit in drying the solid in B.t.u./min. E_T values for Eq. 6 were obtained from Eq. 7

$$E_T = (W)(\lambda) \quad \text{(Eq. 7)}$$

where W = water evaporated per minute in drying solid to equilibrium M.C.—Kg./min., λ = latent heat of vaporization for water—B.t.u./Kg. The values of E_A required for Eq. 6 were calculated from Eq. 8.

$$E_A = (C_p)V\rho\Delta T \quad \text{(Eq. 8)}$$

where C_p = heat capacity of air—$\dfrac{\text{B.t.u.}}{\text{lb.}\,^\circ\text{F.}}$,

V = air velocity through dryer—C.F.M. $\dfrac{(\text{ft.}^3)}{(\text{minute})}$ (air velocities can be approximated from the manufacturer's stated operating values); ρ = density of air—$\dfrac{\text{lb.}}{\text{ft.}^3}$; ΔT = temperature gradient between ambient air temperature and averaged temperature of heated air at inlet to drying compartment—°F.

perature during drying, thereby reducing color bleeding and case hardening effects, and facilitating the handling of heat sensitive materials.

4. Decreased handling costs resulting from simplified loading and unloading operations.

5. Simplification of tablet manufacturing procedures by the possible elimination of grinding steps. Opportunities may also exist for blending lubricants and other materials into dry granulations directly in the fluidized bed.

Fluidized drying beds of small capacity appear to have further significant usefulness in research operations for routine testing of new tablet formulas on a rapid basis.

Because of the extensive application in other industries of the fluidized bed drying technique, it is reasonable to assume that the conclusions of this report are not limited to the prototype granulations actually tested. The Glatt dryer used in this study represents a significant improvement over conventional tray dryers. The present data suggest, however, that the drying performance of this unit is not as high as that which can be expected from fluidized bed dryers.

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Psychophysical Concept of Color

By ALLAN M. RAFF

Color has been defined as a psychophysical concept by the Committee on Colorimetry of the Optical Society of America. The concept of the Commission Internationale de l'Eclairage (C.I.E.) standard human observer is discussed, which illustrates the color sensitivity curves on which the C.I.E. color triangle is based. Examples are given which show the variance in results that occur in color stability studies when a "standard observer" is used as opposed to an evaluation made solely on the basis of spectrophotometric data. Several standard techniques for evaluating small color differences are reviewed.

WITHIN the last several years the stability studies of pharmaceutical products have become increasingly more sophisticated. The use of kinetics, cyclization of temperatures, and more advanced forms of instrumentation are just a few of the things that have brought the study of the chemical stability of pharmaceutical products to a very high level.

This same high level has yet to be reached in the matter of color stability studies. This deficiency may be due to two causes. The first cause has been a lack of the need for such studies until recent times. Until the FDA decertified some dyes and indicated a prospect for the decertification of several more, the pharmaceutical formulator had a relatively easy task. He had only to choose the most stable dye from a long list of dyes and color his product. Today he has the task of determining which of several very photosensitive dyes is the most stable. He must also make matches of colors heretofore achieved with one dye (perhaps now decertified), now with different colorants. The second cause of the deficiency noted is due in a very large part to a complete misunderstanding of the concept of color as it should be used in pharmaceutical stability studies.

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Color has been defined as a psychophysical concept by the Committee on Colorimetry of the Optical Society of America. The concept of the Commission Internationale de l'Eclairage (C.I.E.) standard human observer is discussed, which illustrates the color sensitivity curves on which the C.I.E. color triangle is based. Examples are given which show the variance in results that occur in color stability studies when a "standard observer" is used as opposed to an evaluation made solely on the basis of spectrophotometric data. Several standard techniques for evaluating small color differences are reviewed.

WITHIN the last several years the stability studies of pharmaceutical products have become increasingly more sophisticated. The use of kinetics, cyclization of temperatures, and more advanced forms of instrumentation are just a few of the things that have brought the study of the chemical stability of pharmaceutical products to a very high level.

This same high level has yet to be reached in the matter of color stability studies. This deficiency may be due to two causes. The first cause has been a lack of the need for such studies until recent times. Until the FDA decertified some dyes and indicated a prospect for the decertification of several more, the pharmaceutical formulator had a relatively easy task. He had only to choose the most stable dye from a long list of dyes and color his product. Today he has the task of determining which of several very photosensitive dyes is the most stable. He must also make matches of colors heretofore achieved with one dye (perhaps now decertified), now with different colorants. The second cause of the deficiency noted is due in a very large part to a complete misunderstanding of the concept of color as it should be used in pharmaceutical stability studies.

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METHODOLOGY

The increased need of color stability studies has brought about a corresponding increase in the application of scientific methodology to this field. There have been several studies (1–6) reported which utilize such techniques as kinetics, advanced instrumentation, and reproducible methods of fading pharmaceutical dosage samples. Investigations such as these have alleviated one of the causes of unsophisticated color stability studies. However, even these investigations may be inconclusive because none of them have treated color as a psychophysical concept.

If we stop short of this concept, *i.e.*, if we confine all of our color studies to an instrumental level, then we have not, in reality, made a study of color stability.

Concepts of Color.—As defined by the Optical Society of America (7), "Color consists of the characteristics of light other than spatial and temporal inhomogeneities; light being that aspect of radiant energy of which a human observer is aware through the visual sensations which arise from the stimulation of the retina of the eye." This concept is reiterated in one form or another in other references in the field of color measurement (8–13).

Nickerson (14) states that: "It is true that a spectral distribution curve may serve to define unambiguously the *stimulus* for the color of a light source, but the spectral-reflectance or spectral-transmittance curve of an object is not sufficient to define even the stimulus for its color. Its spectral properties must be combined with those of the light source under which it is to be viewed before even the stimulus for the object's color is defined. To define its *color* the concept of an observer must also be introduced."

The *Ciba Review* (15) expresses this concept: "Measurement of the spectral reflection curves of typical colored surfaces shows, as we should expect, that a red surface has a high reflection at the red end of the spectrum, but a low reflection in the green and blue. A green surface has its maximum reflection in the green, and a blue surface in the blue. Consideration of the reflection curve for a yellow surface, however, shows that *this correlation is of limited application*, since a yellow surface is nearly as efficient in reflecting the green and red wavelengths as it is the narrow band of yellow wavelengths around 0.58 μ. *Clearly, an adequate interpretation must take account of the combined effect in the eye of all the wavelengths reflected by the surface.*"[1]

This consideration of color as a psychophysical concept is of more than academic interest in pharmaceutical formulating and in the resultant color stability studies of these products. Colorants are added to various pharmaceutical dosage forms, unlike the active ingredients, primarily for their esthetic effect and as a means of identification. When one assays for the active constituent in a product and it is found to be only 50% of theory then this may be, with a fair degree of certainty, interpreted to mean that there will only be 50% of the desired therapeutic response. However, if one determines by a spectrophotometric assay that a green colorant has decreased in concentration by a numerical value of 10% and a yellow colorant has also decreased by an equal amount, one cannot say without performing certain mathematical transformations that these two colorants will *appear* to a *human observer* to have faded equal amounts. Since the relationship between the visual appearance of colors is what one is really attempting to establish when color stability studies are done, it is of the utmost importance that the concept of the human observer be brought into consideration.

[1] Italics those of the author.

EXPERIMENTAL

In order to illustrate the errors that may occur when we attempt to evaluate color solely on the basis of spectrophotometric data, the following study is presented.[2] Test panels containing pigments were exposed to sunlight, and after several months exposed and unexposed panels were measured on a G.E. recording spectrophotometer equipped with a Librascope automatic integrator. Color differences were calculated using the Godlove formula (16) and the results expressed in N.B.S. units (10, 17, 18, appendix of this article[3]).

Figures 1–5 illustrate the reflectance measurements on control and exposed panels of violet, green, yellow, orange, and red pigment colorants, respectively. Figures 6 and 7 are the reflectance curves of Figs. 2 and 3, respectively, mathematically transformed into "absorbance" units.

It may be seen from these figures and the compilation in Table I that no correlation can be made,

TABLE I.—REFLECTANCE AND "ABSORBANCE" DIFFERENCES *vs.* COLOR CHANGE

Color	Color Change N.B.S. Units	Per Cent Difference in Reflectance[a] at Max.	at Min.	Difference in Absorbance[a] at Max.
Violet	5	1	1	
Green	5	0	5	0.068[b]
Yellow	6	6	1	−0.028[b]
Orange	4	5	1	
Red	5	1	1	

[a] Value of control minus that of exposed. [b] Absorbance units.

by a visual inspection of the curves, between a change in reflectance (or so-called absorbance) and the change in color that one will perceive.

All five of these colorants have changed to different degrees, but their appearances are essentially the same in terms of visual perception.

The pigments chosen yielded relatively simple curves. Other colorants and mixtures of colorants that display more than one maximum or exhibit metamerism would be even more illustrative of this lack of correlation.

In view of this demonstrated lack of correlation between reflectance curves and the color perceived by a human observer, the author believes that it is the responsibility of the investigator reporting on

[2] These test panels and the evaluation of their color differences in N.B.S. units were a part of a study made by Vincent C. Vesce, while technical director of Harmon Colors, National Aniline Division, Allied Chemical Corp. (19). I wish to thank Mr. Max Saltzman of National Aniline Division of Allied Chemical Corp. for allowing the use of these data and his very generous giving of his time and assistance to the author.

[3] A brief review of the techniques useful to those engaged in color studies is presented in an appendix to this article and is available from the author.

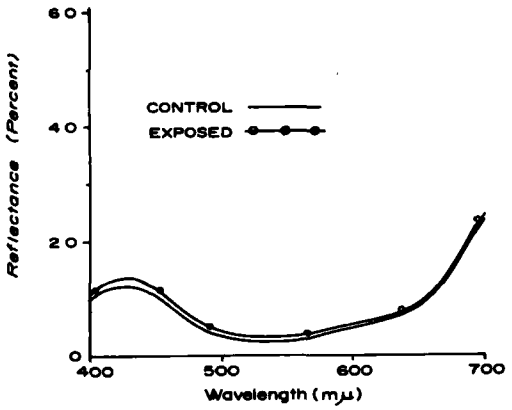

Fig. 1—Reflectance measurement of a violet pigment; Color Index No. 60016; color change is 5 N.B.S. units.

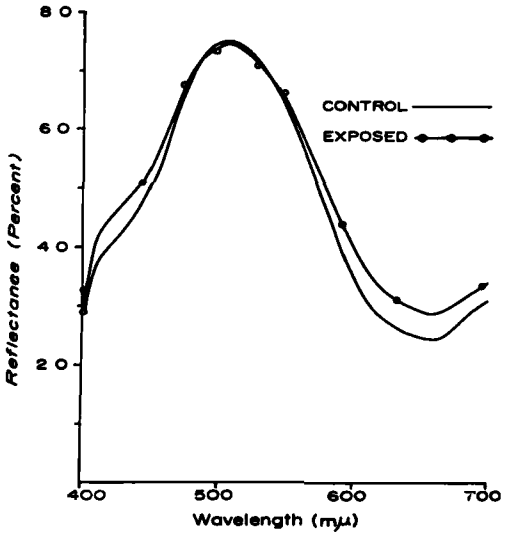

Fig. 2.—Reflectance measurement of a green pigment; Color Index No. 74120; color change is 5 N.B.S. units.

color studies to prove that where it is utilized, a single wavelength determination is valid in each and every case studied. This is not to say that a single wavelength cannot be utilized to follow the course of a colorant change, but it must be substantiated by sufficient data to prove its validity. This method of using a single wavelength determination has been used, for example, by McLaren (20). He states, however, "This method of determining the degree of fading is only very rough; greater accuracy could have been achieved if the Kubelka-Munk equation or the best of the American colour difference formulas had been used. Such methods would have taken very much longer, however, and did not appear essential in view of the elementary nature of the investigation."

A failure to transform spectrophotometric data into some system which takes into account the sensitivity of the eye in conjunction with a specific light source will also, in some instances, invalidate those studies that attempt to rank different colorants with regards to their resistance to fading.

Other Experiments.—Urbanyi, *et al.* (3), measure the reflectance of opaque objects in terms of absorbance, yet absorbance is, by definition, the negative log of transmittance.

In dealing with transmission data the values of the various concentrations follow Beer's law. Absorbance ($-\log T$) is directly proportional to concentration. For reflectance work, two ordinate scales are in use. The linear scale is the most common, but has the same limitations as the linear scale in transmittance work. Reflectance data at various concentrations follow an entirely different scheme which may be termed the K/S rule. Kubelka and Munk

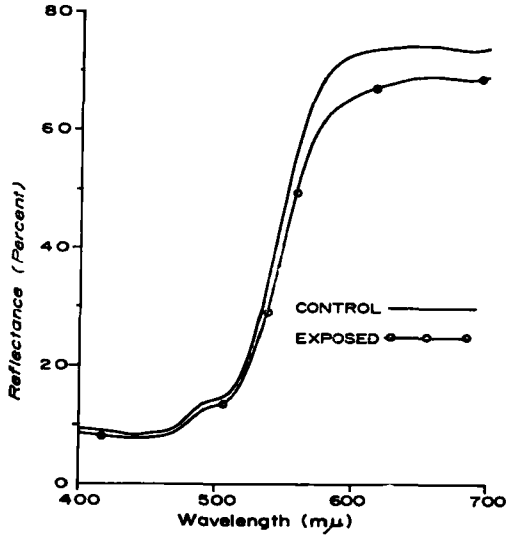

Fig. 3.—Reflectance measurement of a yellow pigment; Color Index No. 70600; color change is 6 N.B.S. units.

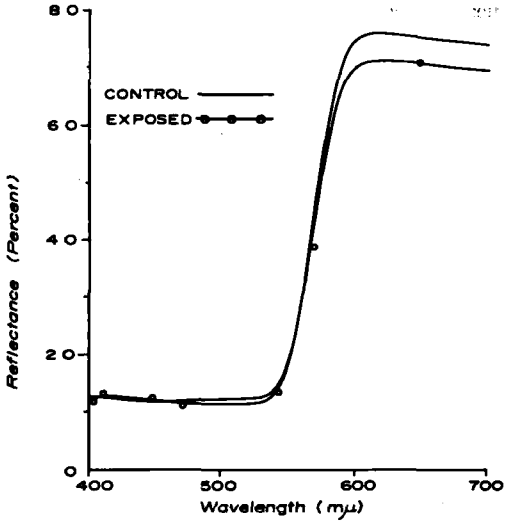

Fig. 4.—Reflectance measurement of an orange pigment; Color Index No. 11725; color change is 4 N.B.S. units.

(10, 21, 22) defined monochromatic reflectivity as

$$K/S = \frac{(1 - R_\infty)^2}{2R_\infty}$$

where:

$$R_\infty = 1 + (K/S) - [(K/S)^2 + 2(K/S)]^{1/2}$$

K = absorption coefficient or fraction of the incident energy lost by absorption per unit thickness of material, while S = scattering coefficient or fraction of the incident energy lost due to scattering per unit thickness of material.

This theory has been extended by others, showing that within limits, the dye and a matrix material absorb and scatter light independently of one another and that the ratio K/S for the dye (as distinct from K/S for the matrix material) bears a proportionality to the amount of dye present.

Urbanyi further utilizes a tristimulus technique measuring the color of opaque objects at only three wavelengths. The tristimulus values are normally computed by integrating the product of the ordinates

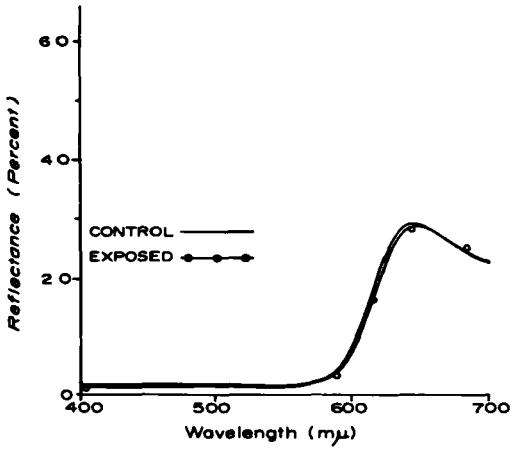

Fig. 5.—Reflectance measurement of a red pigment; Color Index No. 15865; color change is 5 N.B.S. units.

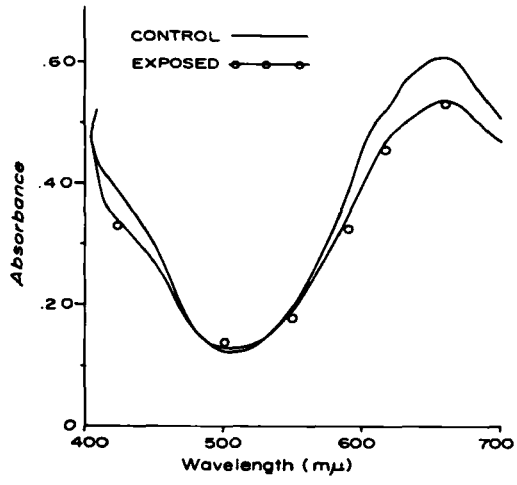

Fig. 6.—Reflectance data of Fig. 2 mathematically transformed into "absorbance" units and replotted.

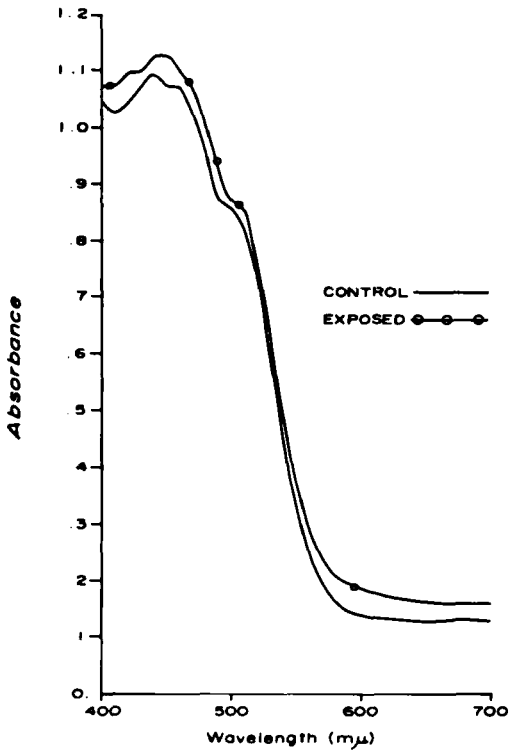

Fig. 7.—Reflectance data of Fig. 3 mathematically transformed into "absorbance" units and replotted.

of one of the standard color mixture curves multiplied by the energy in the corresponding wavelength regions of the spectrum of the sample of radiant energy. If the sample of radiant energy is that reflected from a surface, the spectral composition of the reflected energy is given by the products at each wavelength of the spectral composition of the incident energy and the corresponding radiant reflectance of the surface. This product can be employed as the spectral composition of the reflected energy in the calculation of the tristimulus values of the color of the light reflected from the surface. The integration may be carried out by use of any convenient method of numerical integration (consult appendix).

Lachman, *et al.* (4), ranked dyes in the order of decreasing light stability by improperly using an inadequate abridged selected-ordinate method and the results were stated in "absorbance" units. Furthermore, they failed to establish the validity of the single wavelength type of measurement and their basis of ranking did not take into account the sensitivity of the human eye.

Garrett and Carper (1) determined the color stability of a multisulfa preparation by decanting the clear liquid containing the dye and determining the spectrophotometric absorbance of the dye. While their results and conclusions drawn may be perfectly correct, they nevertheless failed to show that there was a correlation between the dye content *in vitro* and the color of the multisulfa preparation. Since in pharmacy the primary purpose in coloring a product is its total visual effect, then it would seem to be most important to measure color as

perceived, not some other attribute that may or may not correlate with it unless the correlation has been previously rigorously established.

Vickerstaff and Tough (23) discussed the uncertainties of equating fading with the destruction of the dyes involved. They stated that a 50% fade need not involve the destruction of 50% of the dye. The only type of substrate to which such a criterion could be applied with justification is the dyed transparent film, where dye concentrations can be estimated from transmission measurements, and the true reduction in concentration on fading determined.

In another publication of Lachman, *et al.* (5), the comparative light fastness of several water-soluble dyes and their corresponding lakes were evaluated. To make a comparative light stability study of tablets colored with water-soluble dyes and lakes of the same dyes, the concentration of dye in the amount of lake used for the tablets was calculated, and the same quantity of dye was used for the tablets colored with the water-soluble dye. The results of this study indicated that the photostability of the lakes was less than in those of the dyes in the systems studied.

While these results may be correct it should be pointed out that the lakes and dyes were in the tablet formulas in equal dye contents, not equal tinctorial content. The color of the tablets, both before and after fading, is the significant matter, and not the dye contents. Perhaps a more valuable study could have been achieved by starting with the lakes and dyes having the same appearance and then noting their fading with time and light. The *Ciba Review* (15) states, "Perhaps the greatest emphasis should be placed on the recognition that colour is subjective and that colour appearance is not just a matter of spectral absorption and reflection."

Some of the anomalies that have been discussed in the pharmaceutical literature with regard to the light fastness of dyes and lakes varying in different formulations can be, in part, resolved by the following concepts.

Smith and Stead (24) contend that the light fastness of a pigment has no significance and that it is only the light fastness of a pigmentation that can be measured and has any value. In their study they went on to describe the wide differences in light fastness shown by using the same pigment at the same concentration in different media. They further stated: "All that can fairly be said of pigments is that Pigment A used according to Formulation B at Concentration C in Medium D exposed to Light Source E for Time F under Humidity G and Atmospheric Conditions H at Temperature I has a Fastness X relative to an arbitrary standard. This is a *reductio ad absurdum* mode of presenting the idea, but the absurd in this case is strictly true."

Schwen and Schmidt (25) substantiated this in their study. They said: "In the light of present knowledge it is now quite clear that the system comprising dye and fibre together—or, rather, dye, fibre, and further components—is necessary to define the light fastness of a particular dyeing. One cannot quote the light fastness of a dye alone."

SUMMARY

If those working in the field of pharmacy will realize that it is not the colorants but the color that is the primary objective in coloring pharmaceutical products, then they will be able to take advantage of a vast amount of research that has been done by workers in the fields of textiles, plastics, pigments, dyes, ceramics, films, and paints. The government, through the Bureau of Standards and the Department of Agriculture, has made very basic and rewarding contributions to the science of color. The Optical Society of America and the Inter Society Color Council are just two of the many organizations whose research, when utilized by pharmacy, could put us far ahead of where we are today with regards to the study of color in all of its facets.

To date there has been no study conducted in the pharmaceutical field which treats color as the psychophysical phenomenon that it is. Until this is done all results and conclusions will be open to question.

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Adaptation of Commercial Viscometers for Special Applications in Pharmaceutical Rheology I

The Brookfield Viscometer

By JOHN H. WOOD, GREGORY CATACALOS, and S. V. LIEBERMAN†

The adaptation of the Brookfield LVT and RVT viscometers to absolute rheometers by the use of a fixed cup and bob system is described. The resultant instruments, providing shear rates in the 0.1 to 15 seconds^{-1} range, supplement the Drage Epprecht rheometer in the next two lower decades of shear. The converted LVT was used to evaluate the apparent shears found with the conventional disk spindles of the Brookfield RVF. It is shown that the resultant shear is an integration of the Newtonian and non-Newtonian components. Thus, although the use of several speeds with such spindles does give indications of pseudoplastic behavior, the shear dependency is not fully characterized.

ONE OF THE most widely available viscometers for general control use has been the Brookfield Synchro-Electric viscometer in one of its several models. For routine control measurement, it is frequently customary to use only the most suitable speed for measurement with a given spindle. Most spindles are of the disk type rather than the bob or rod. As a result of this general type of usage, the instrument has tended to be somewhat in disrepute among practicing rheologists. There have been several attempts (1–3) to apply the use of the several available speeds of the instrument, four in the F models and eight in the T models, to the study of non-Newtonian systems. However, these have been confined to the use of the normally supplied Brookfield disk spindles, and hence have been limited by the nondefinable shear-rates used.

Since this instrument in the T model has very slow speeds available, it seemed desirable to attempt to fit it with a series of cups and bobs such that reasonably absolute shear measurements might be made in the low shear-rate range.

EXPERIMENTAL

Initially a Brookfield model LVT viscometer was equipped with cups *A* and *B* of internal diameters 2.54 and 0.852 cm., respectively, attached rigidly to the viscometer by a twist plate identical to that normally used to mount the guard ring. (See Fig. 1.) The Nos. 1 and 4 spindles as normally supplied are straight geometric cylinders. Similar geometric spindles are available for Nos. 2 and 3 on order from Brookfield Engineering Corporation, Stoughton, Mass. The pertinent dimensions are given in Table I. All are flat bottomed and have a shaft diameter of 3.0 mm.

It was intended that the use of this instrument would be primarily to complement the Drage Epprecht rheometer by supplying the lower shear-rate range. For this reason Newtonian shear rates were calculated in accordance with the standard

TABLE I.—DIMENSIONS OF CYLINDRICAL BROOKFIELD LVT SPINDLES

Dimension	Brookfield Spindle Number 1	2	3	4
Diameter, cm.	1.9	1.0	0.6	0.3
Length, cm.	6.5	5.4	4.3	3.1

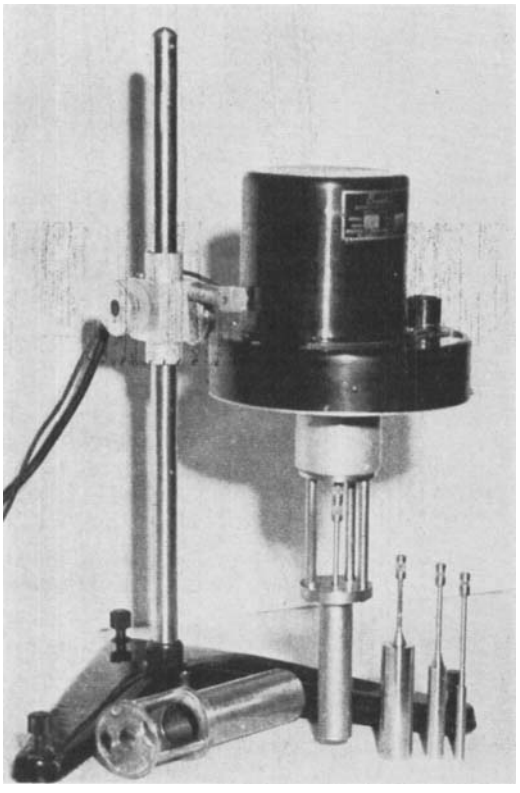

Fig. 1.—Brookfield LVT with constructed cups and cylindrical spindles.

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practice for that instrument (4). The torque factor was calibrated experimentally from the slope of the plot of the scale reading of the instrument *vs.* calculated shear rates for all suitable National Bureau of Standards calibration oils. These oils are all Newtonian in the range of shears used here, so that straight line plots were obtained for each bob for each oil used. This method previously had provided torque factors for the Drage identical to those cited by the manufacturer.

Since the spindle mounting of the Brookfield is not rigid but quite free-floating, close cup-to-bob clearances are not practical. In types of non-Newtonians with which the instrument has been used for the last 3 years, the use of the *A* cup with spindles 1 and 2, occasionally 3, has been the general rule. For those systems for which the two thinnest bobs, 3 and 4, are needed it is often not practical to fill the smaller cup without the use of excessive disturbing shear of the sample. We intend to extend our usage of the smaller bobs by the insertion into the filled *A* cup of sleeves similar in design to those now available with the Drage Epprecht rheometer. These reduce the cup-to-bob ratio, and incidentally raise the shear rate used. These sleeves can be inserted into filled cups with very little shear disturbance.

Most applications of this instrument, as with the Drage, involve direct comparisons between samples measured in identical manners. This is particularly true of its use in routine aging comparisons.

For heavier consistency systems we have similarly adapted a Brookfield model RVT using the same bobs and the large cup only. Here the cup is mounted by an adapter plate which connects by the mounting screws normally used to bolt on the guard ring. The relative spring moments of the LVT and RVT models as given by the manufacturer are 6.737 and 71.97 dyne cm./division, respectively.

Apparent Shear with Regular Brookfield RVF Spindles.—Frequently it has been desirable to know under what equivalent shear routine control type Brookfield RVF readings were being taken. Accordingly six pseudoplastic materials were chosen of varying viscosity characteristics as shown in Table II.

TABLE II.—VISCOSITY CHARACTERISTIC OF TEST SUBSTANCES AS FUNCTION OF SHEAR RATE

Test Substance	Apparent Viscosity (Point to Origin) D = 0.1 sec.$^{-1}$	D = 1 sec.$^{-1}$	Cps. D = 10 sec.$^{-1}$
1	36,000	14,000	2,200
2	42,000	17,000	3,700
3	30,000	5,000	940
4	22,000	3,900	870
5	13,000	4,400	920
6	66,000	14,000	3,500

All RVF spindles which would give readings were also used with each lotion. The multiplying factors appropriate to each spindle as supplied by the manufacturer were used to calculate apparent viscosities. Plots were then made for each liquid against the relative Newtonian apparent shear rate. An example is shown in Fig. 2. The reference point chosen was the No. 7 spindle at 2 r.p.m. This has the highest multiplying factor, 20,000. This was given a relative Brookfield shear rate of 1*X*. The relative shear rates were assumed to vary with the multiplying factor. Thus spindle No. 3 at 20 r.p.m. with a multiplying factor of 50 was given a relative shear of 20,000/50 or 4000*X*. The shear rate from the rheogram necessary to give an apparent viscosity equivalent to that observed using the Brookfield multiplying factor was taken as the Spindle Equivalent Shear Rate for that operating condition.

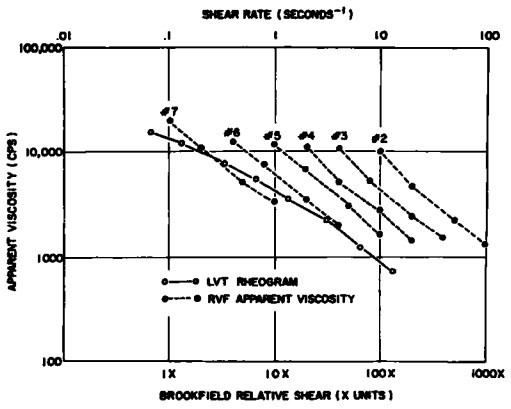

Fig. 2.—Comparison between conventional Brookfield RVF readings at each speed for the various spindles and a rheogram from the modified LVT for the same pseudoplastic lotion.

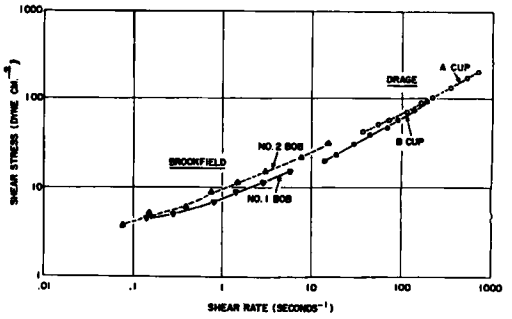

Fig. 3.—Rheograms run with the modified Brookfield LVT and with the Drage Epprecht viscometer for a pseudoplastic lotion.

RESULTS AND DISCUSSION

Rheology.—In the calculation of shear rates for cup-and-bob combinations where the radius ratio departs significantly from unity, the choice of the method of calculation is a major problem. Fischer (5) and Andrade (6) have tried different approaches to the integration of the average shear rate. Since the Newtonian viscosity is independent of the integration procedure used, the shear stress term must bear the same averaging method. It was therefore decided to take the direct approach, namely, to use the simple Newtonian shear-rate formula and, by the empirical calibration, include any necessary correction factor into the torque term.

This is justified because a multispeed instrument is of no value for Newtonian systems, but is of particular need for pseudoplastic ones. In such systems, having as they do exponential torque-shear rate dependencies, it is no longer possible to simply relate the shear gradient to the system geometry since the nature of the shear dependency enters into

TABLE III.—APPARENT SPINDLE EQUIVALENT SHEAR RATES FOR BROOKFIELD RVF SEC.$^{-1}$

Spindle Speed, r.p.m.	Brookfield Spindle Number					
	7	6	5	4	3	2
2	0.03–0.22	0.11–0.33	0.09–0.50	0.10–0.50	0.11–0.54	0.19–0.60
4	0.12–0.40	0.09–0.70	0.5 –1.1	0.5 –1.1	0.7 –1.2	0.8 –1.2
10	0.6 –1.4	1.3 –2.5	1.9 –2.9	2.1 –2.8	2.6 –3.2	3.2 –3.6
20	1.5 –3.8	3.7 –4.8	4.3 –5.3	4.3 –5.6	4.4 –6.2	6.0 –7.4

the observed gradient. In this case, for absolute measurements, all calculations must be reduced to the walls of the measuring system (7). In the extrapolated limit, any equations should lead to the same values for apparent shear rate and torque at the wall. It is intended that such a treatment be the subject of a later paper.

Figure 3 shows the results obtained for the measurement of an antiperspirant lotion at 25° using the Nos. 1 and 2 bobs of the Brookfield and the *A* and *B* cups of the Drage rheometer. It is apparent that this is a pseudoplastic system. The variety of cup and bob ratios used here partially justifies Fischer's statement (5) that "For non-Newtonian liquids . . . the mean value of η is found experimentally to be approximately the same for large and small clearances, so that the effect of variable rate of shear apparently balances out."

The Brookfield LVT modified in the manner shown compliments the Drage Epprecht viscometer by providing for the two to three decades of shear lower than otherwise available. This modification thus provides an economical low-shear rheometer suitable for measurements in the range normally found for tipping and slow pouring from a bottle of rather heavy lotions (8). Thus the rheological study of their aging in a useful shear rate range is facilitated. The use of this instrument in the study of Veegum suspensions is given elsewhere (9).

Apparent Shear of Brookfield RVF Spindles.—Table III gives the ranges found for the Equivalent Shear Rates for the various spindles in use with the Brookfield RVF. It will be noted in Fig. 2, that with the exception of the No. 7 spindle (which has no disk) the apparent viscosity at each speed was surprisingly independent of the bob used to measure it. (Note the correspondence of the serial spots for each bob.)

The slopes of the bob lines are relatively parallel, but do not in all cases parallel the shear-dependency line of the rheogram. No absolute correlation can be made between apparent Brookfield readings for a given speed and the viscosity of a non-Newtonian. Nevertheless, the ranges of apparent shear rate are narrow enough that we consider we can predict satisfactorily from the rheogram of a given system what its apparent Brookfield viscosity will be.

Thus readings obtained by a conventional Brookfield RVF can be empirically equated to the values found from a conventional rheogram. However, the apparent viscosity value does depend on the spindle used, as does the apparent shear dependency. The fact that such correlation is possible obviously follows from the reasonable success the instrument has had in process monitoring.

With such a low-shear rheometer, equivalent types of calibrations may be made of shear rates of other nondefinable measuring systems.

SUMMARY

A readily constructed attachment to convert a Brookfield viscometer of the T type to an absolute rheometer is described. Its application in the case of the Brookfield model LVT to give a rheometer of shear rate range 0.1 to 15 sec.$^{-1}$ is described.

The equivalent shear rate obtained by conventional Brookfield RVF with disk spindles was determined by equating the viscosities obtained to the rheogram. It is found that, since the spindle integrates the non-Newtonian with the Newtonian component, the use of the multispeed determination, as described in the literature, gives a useful but an incomplete idea of the non-Newtonian character.

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Effect of Surface-Active Agents on the Extraction of Belladonna Herb

By G. P. SRIVASTAVA and T. N. CHADHA

The effect of two surface-active agents, Tween 20 and Tween 80, on the extraction of belladonna herb (*Atropa belladonna*) by percolation and mechanical agitation processes using 70% alcohol and water as solvents has been studied. The concentration of the surfactants in the solvents was uniformly 0.2%. These surfactants have been shown to potentiate the solvents leading to a more complete and expedient extraction of the drug. Seventy per cent alcohol and water containing 0.2% Tween 20 extracted the drug better than the unpotentiated solvents. Tween 80 gave better results than Tween 20 when used in the extraction by mechanical agitation. In the percolation process only Tween 20 was used.

In recent years some novel methods of drug extraction have been suggested. Bay and Gisvold (1) worked out a method for extracting digitalis leaves by disintegrating them in a Waring Blendor in presence of water as menstruum. Greco and Dumez (2) used a pressure cooker for extracting vegetable drugs like nux vomica, belladonna, hyoscyamus, stramonium, cascara, and wild cherry. Changes in the use of solvents have also been suggested. Carkhuff and Gramling (3) used various furans for the extraction of belladonna. Campo and Gramling (4, 5) extended the use of furans to the extraction of defatted ergot and cinchona. Dean, *et al.* (6), made use of a colloid mill for preparing tinctures of belladonna and stramonium. Head, *et al.* (7), worked on the ultrasonic extraction of *Cinchona succirubra* using various solvents. Bose, *et al.* (8), also employed this method in the extraction of *Rauwolfia serpentina*. Different workers have shown advantages of their respective methods.

The use of surface-active agents in the extraction of vegetable drugs was suggested by Butler and Wiese (9), who successfully employed such agents for extracting belladonna, hyoscyamus, cinchona, and ipecac. They used several surfactants and extracted the drugs by the official percolation method. Brochmann-Hanssen (10) followed the use of surfactants for the extraction of cinchona and ipecac. These investigations induced the present authors to undertake the inquiry of the effect of two surface-active agents, Tween 20 (polyoxyethylene sorbitan monolaurate) and Tween 80 (polyoxyethylene sorbitan mono-oleate), not so far studied by the previous workers, on the extraction of belladonna herb (*Atropa belladonna*), an important drug of the Indian Pharmacopoeia. The above two surfactants belong to the nonionic group and are practically nontoxic (11, 12).

A surfactant concentration of 0.2%, usually employed in pharmaceutical formulations, was used in 70% alcohol and in water. Extractions were done by percolation and mechanical agitation. Throughout this study we employed 70% alcohol where alcohol was utilized and maintained the concentration of the surfactants at 0.2%.

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EXPERIMENTAL

A sample of belladonna herb (*A. belladonna*) powdered to No. 40 and assayed by the method of the Indian Pharmacopoeia of 1955 (13), was used all through the present work. The average result of six readings of assay was 0.08782% w/w of alkaloids calculated as hyoscyamine.

Extraction by Percolation.—In the first series of experiments 100 Gm. of the drug powder (No. 40) was extracted by the percolation process in the following ways: (*a*) simple percolation using alcohol as menstruum (control for comparison); (*b*) percolation without 24-hour maceration using alcohol containing Tween 20 for moistening only and alcohol for the rest of the process; (*c*) simple precolation using alcohol containing Tween 20 for moistening as well as for maceration and alcohol for the rest of the process; and (*d*) percolation without 24-hour maceration using alcohol containing Tween 20 for the entire process.

In every case the same size percolator was used and the rate of percolation was maintained at 30 drops per minute. A 700-ml. quantity of the percolate was collected in five fractions—three 100 ml. and two 200 ml. Each fraction was assayed for its alkaloidal content and from the data thus obtained the progressive percentage of alkaloids extracted from 100 Gm. of the drug was calculated. Three sets of experiments were done in each case and the average values are recorded in Table I. The total solids (T.S.) extracted from 100 Gm. of drug was determined and the solvent selectivity index (s.s.i. = alkaloids × 100/total solids) was calculated therefrom. These values are also noted in Table I.

Table I.—Results of the Extraction of Belladonna Herb by the Percolation Process

Fraction No.	Progressive Percentage of Alkaloid Extracted (a)	(b)	(c)	(d)
1	55.7	47.3	51.3	53.2
2	83.8	74.4	80.5	81.2
3	93.2	84.6	89.0	92.1
4	97.7	89.6	94.4	98.3
5	100.4	91.4	96.6	100.6
s.s.i.	0.662	0.625	0.570	0.632

It is clear that complete extraction of alkaloids from belladonna herb is effected by percolation without 24-hour maceration when Tween 20 is incorporated in the total volume of alcohol used as menstruum. The efficiency of extraction procedure *d* is similar to simple percolation with 24-hour maceration, and plain alcohol procedure *a*. The solvent selectivity, however, is slightly reduced in this case. The other modifications adopted, *b* and *c*, have not proved successful.

Extraction by Mechanical Agitation.—In order to have more controllable conditions and obtain comparable results, extraction by mechanical agitation with or without maceration was used in the following series of experiments. For this purpose 20 Gm. of drug powder (No. 40) was taken with 150 ml. of menstruum in a well-closed 250-ml. bottle and agitated in a mechanical shaker at the rate of 200 strokes per minute. At the end of the specified time the clear extract was decanted off. Duplicate samples were prepared in each case and each sample was assayed twice for alkaloids taking 50 ml. of extract each time; total solids were also determined. Total alkaloids and solids present in the total volume of menstruum added (150 ml.) were calculated. This was done to find out the percentage of the alkaloids in 20 Gm. of drug (calculated value = 0.0176 Gm.) taken up by the total volume of the menstruum at the end of the process. In this way the results obtained were rendered comparable.

The effect of the duration of time of agitation was first determined; 20 Gm. of the drug and 150 ml. of alcohol containing Tween 20 were first macerated for 24 hours and then shaken for 5, 10, 20, and 30 minutes. As a control for comparison, 20 Gm. of the drug was also extracted with plain alcohol and agitated for 30 minutes by this method. The results are recorded in Table II.

TABLE II.—RESULTS OF EXTRACTION OF BELLADONNA HERB BY MECHANICAL AGITATION AFTER 24-HOUR MACERATION

Agitation, Minutes	Menstruum Used	Alkaloid Extracted, %	s.s.i.
5	Alcohol with Tween 20	67.9	0.420
10	Alcohol with Tween 20	71.7	0.442
20	Alcohol with Tween 20	83.2	0.510
30	Alcohol with Tween 20	90.9	0.535
30	Alcohol (control)	46.1	0.294

The results in Table II show evidence of the superiority of the extraction of alkaloids from belladonna herb with alcohol containing Tween 20 over the extraction with plain alcohol; the extraction with agitation for 30 minutes in the former case is nearly double (90.9%) of that in the latter (46.1%). Even agitation for 5 minutes with the potentiated menstruum extracts nearly one and a half times more alkaloids than in the control. It is interesting to note that the solvent selectivity index is also higher in the case of the potentiated menstruum.

In the following experiments the 24-hour maceration before agitation was eliminated and 20 Gm. of the drug was agitated with 150 ml. of solvent. The time of shaking was increased as shown in Table III. Three types of solvents were used: (i) plain alcohol as control for comparison, (ii) alcohol containing Tween 20, and (iii) purified water containing Tween 20. The results obtained are given in Table III.

TABLE III.—RESULTS OF EXTRACTION OF BELLADONNA HERB BY MECHANICAL AGITATION WITHOUT MACERATION

Agitation, Minutes	Menstruum Used	Alkaloid Extracted, %	s.s.i.
30	Alcohol with Tween 20	26.4	0.296
60	Alcohol with Tween 20	34.4	0.377
90	Alcohol with Tween 20	73.9	0.480
120	Alcohol with Tween 20	97.6	0.595
60	Water with Tween 20	53.1	0.143
120	Water with Tween 20	66.4	0.187
120	Alcohol (control)	42.5	0.313

It is clear that 2 hours of agitation of the drug with alcohol containing Tween 20, without maceration, extracts it almost completely (97.6%), purified water containing Tween 20 extracts 66.4% of the alkaloids, while plain alcohol under similar conditions extracts only 42.5% of the alkaloids. It is also significant to note that Tween 20 in alcohol gives a much higher value for the solvent selectivity index than the value obtained in the case of water.

Having examined the effect of Tween 20, some experiments were also done with Tween 80, both in alcohol and water. Extraction of 20 Gm. of the drug was done by mechanical agitation without maceration in 150 ml. of the menstruum. Duplicate samples were prepared and each sample was assayed twice. The results are given in Table IV. For the sake of ready comparison, the values of per cent alkaloids extracted by alcohol and water both potentiated separately and severally with Tween 20 and Tween 80 are tabulated in Table V.

TABLE IV.—RESULTS OF EXTRACTION OF BELLADONNA HERB BY MECHANICAL AGITATION WITHOUT MACERATION

Agitation, Minutes	Menstruum Used	Alkaloid Extracted, %	s.s.i.
30	Alcohol with Tween 80	72.7	0.519
60	Alcohol with Tween 80	83.87	0.555
90	Alcohol with Tween 80	92.2	0.592
120	Alcohol with Tween 80	100.7	0.640
60	Water with Tween 80	61.5	0.159
120	Water with Tween 80	69.9	0.181

TABLE V.—RESULTS OF EXTRACTION OF BELLADONNA HERB BY MECHANICAL AGITATION WITHOUT MACERATION

Agitation, Minutes	Alkaloid Extracted, %: Alcohol, 70% — Tween 20	Alcohol, 70% — Tween 80	Water — Tween 20	Water — Tween 80
30	26.4	72.7	...	...
60	34.3	83.9	53.1	61.5
90	73.9	92.2	...	...
120	97.6	100.7	66.4	69.9

Tween 80 in the same concentration potentiates both alcohol and water more than Tween 20 does;

and, it also acts better in alcohol than in water. It may be noted that the alcohol incorporating either Tween 20 or Tween 80 extracts the drug almost equally. However, the solvent potentiated with Tween 80 acts more quickly in the beginning as evidenced by the values of per cent alkaloids extracted within short durations. Further, alcohol incorporating Tween 80 acts as a more selective solvent than that containing Tween 20 as indicated by the respective s.s.i. values (0.640 and 0.594).

DISCUSSION

The effect of two surfactants, Tween 20 and Tween 80, on the extraction of belladonna herb has been studied first by the percolation process (Tween 20 only) and then by the mechanical agitation process. It has been found, in both the processes, the solvent (alcohol) is potentiated by adding Tween 20 and permits the preliminary maceration for 24 hours to be eliminated. The purpose of maceration is to allow the drug to imbibe the solvent to facilitate the subsequent extraction. The most apparent effect of the use of the two Tweens in the solvent is that imbibition takes place so quickly that day-long maceration becomes unnecessary and the whole process is expedited.

Though both the surfactants were shown to be effective in potentiating the solvents, Tween 80 was superior to Tween 20 in the mechanical agitation process. This, probably, is due to the presence of the oleate radical, an 18-carbon chain, in the molecule of Tween 80. The laurate radical present in Tween 20 is only a 10-carbon group.

In the percolation process the potentiation of alcohol by Tween 20 has been found not to improve its solvent selectivity, but in the mechanical agitation process there is a marked enhancement. Both Tween 20 and Tween 80 have definitely higher s.s.i. values. The higher the value of this index, the higher will be the selectivity of the solvent, meaning that it extracts the active principles more than it does the other inert constituents.

CONCLUSIONS

Polyoxyethylene sorbitan monolaurate (Tween 20) and polyoxyethylene sorbitan mono-oleate (Tween 80) have been found to potentiate 70% alcohol and water as solvents in the extraction of belladonna herb (*A. belladonna*) by percolation and mechanical agitation processes. Tween 80 acts better than Tween 20 in the latter process. Both these surfactants act better in 70% alcohol than they do in water. In the percolation process the selectivity of the solvent (70% alcohol) is not improved but in the mechanical agitation process it is appreciably enhanced.

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Absorption and Excretion of Sulfadiazine After Subcutaneous Implantation of Disks in Rats

By BERTON E. BALLARD and EINO NELSON†

Thin, cylindrical disk-shaped pellets of sulfadiazine were subcutaneously implanted in rats and free and total urinary sulfadiazine excretion followed. It was possible to relate the mean pellet absorption rate per unit area to the mean excretion rate per unit area and to the fraction of a dose of the drug eventually excreted in the urine.

ABSORPTION of drugs from implants may be studied by following urinary excretion of the unchanged drug, its metabolite(s), or both, by determination of drug blood level, by following excretion of substances such as electrolytes that reflect the drug's action in the body, by studying drug action on a target organ, or by following the decrease in weight of the implants themselves. In previous work it has been shown that the rate-limiting step in absorption after subcutaneous implantation of a number of drugs was the dissolution process at the absorption site (1). The work reported now gives the results of experiments which were conducted to determine the relationship between cumulative urinary excretion of sulfadiazine and acetylated sulfadiazine and change in surface area of thin, cylindrical disks of this material after subcutaneous implantation in rats. A study of this type has not been previously conducted using cylindrical implants; sulfadiazine serves as a readily available and assayable model compound.

PROCEDURE

Thin, cylindrical disks of compressed drug grade sulfadiazine were prepared and subcutaneously implanted in a manner and site described before (1). Four weighed disks were implanted into each of three Sprague-Dawley rats of both sexes designated as A, B, and C, having weights of 210, 335, and 410 Gm., respectively. They were placed in separate glass metabolism cages from which the urine was collected twice daily for 5 days. The urine was assayed for free and total sulfadiazine

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by the Bratton and Marshall method (2). A Bausch and Lomb Spectronic 20 colorimeter was used in the application of the method.

The final mean weights of the disks were corrected for the weight of the proteinaceous "ghost" described by Folley (3) in the following way. A 0.4190-Gm. sample of most of the remains of disks removed from rats A, B, and C was dissolved in dilute ammonium hydroxide. The clear solution was decanted from the "ghost" residues and several portions of distilled water were added and decanted. The small amount of water remaining containing the "ghost" was then evaporated to dryness at room temperature. The residue weighed 3 mg. Thus, the final weight of the disks as yet uncorrected for "ghost" weight was heavy by slightly more than 0.71%. The observed final mean weights in Table I were corrected for the weight of the "ghost."

RESULTS

Table I shows for rats A to C data on the properties and dimensions of the disk-shaped pellets before and after implantation. The initial and final mean disk areas were graphically estimated by a method described previously which used for illustration some of the data given here for rat A (4). The cumulative amounts of free and total sulfadiazine excreted in the urine for the three animals appear in Table II, along with the fraction, f, of total drug recovered in a time infinite in terms of the experiment which was about 147 hours. The nature of the type of cumulative drug excretion curves obtained is shown in Fig. 1 which was constructed from data obtained from rat A. It should be noted that on the cumulative excretion curve only the high points should be considered in the plot, because low points may be due to collection time not corresponding to the last voiding time.

DISCUSSION

There are only a few reports in the literature where the urinary excretion of drug or metabolite(s) have been followed subsequent to drug implantation (5–11). However, except in one of these studies, no attempts were made to relate the absorption rate of implants to urinary excretion rate of drug or metabolite(s) (5).

Theoretically, if the cumulative amount of drug and its metabolite(s) excreted in the urine is plotted against time, one should note a line with a progressively decreasing slope, indicating that the ex-

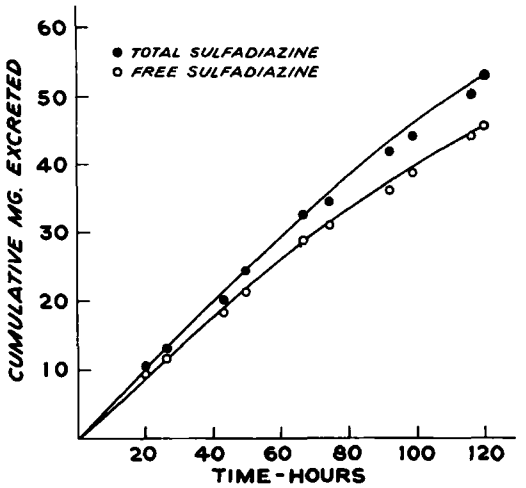

Fig. 1.—Illustrating the nature of the cumulative free and total sulfadiazine excretion *vs.* time curve after subcutaneous implantation of cylindrical disks of drug (Rat A).

TABLE I.—SULFADIAZINE PELLET IMPLANT DIMENSIONS AND PROPERTIES BEFORE AND AFTER IMPLANTATION

Dimension or Property[c]	Rat A[a]		Rat B		Rat C	
	Initial	Final	Initial	Final	Initial	Final
Height, cm.	0.1133	0.0892	0.1196	0.0998	0.1031	0.0846
Diameter, cm.	0.637	0.618	0.637	0.618	0.636	0.621
Weight, Gm.	0.0508	0.0342[b]	0.0531	0.0378[b]	0.0458	0.0319[b]
Estimated area, cm.^{2}	0.864	0.740	0.876	0.761	0.841	0.733
Apparent density, Gm./cm.^{3}	1.407	...	1.405	...	1.401	...
Implantation time (hr.)	...	119.2	...	119.2	...	118.3
$\bar{R}/\bar{A} \times 10^4$ Gm./hr./cm.^{2}	1.74		1.57		1.49	

[a] Data for this animal have been previously presented in connection with a graphic method for estimating mean initial and final implant areas (4). [b] Weight corrected for weight of "ghost." [c] Means of the four thin, cylindrical disks implanted in a given rat.

TABLE II.—CUMULATIVE SULFADIAZINE EXCRETION IN MG. AFTER DRUG IMPLANTATION[a]

Rat A			Rat B			Rat C		
Time, hr.	Free	Total	Time, hr.	Free	Total	Time, hr.	Free	Total
19.2	9.4	10.4	18.8	7.2	9.2	18.3	3.0	3.6
26.2	11.5	12.9	26.0	9.2	11.8	25.8	5.6	7.2
43.0	18.2	20.2	42.8	14.9	20.8	42.2	12.4	15.8
49.2	21.2	24.1	48.8	17.6	24.0	49.1	15.6	20.0
67.3	29.0	32.2	67.2	24.2	31.6	67.1	18.6	24.2
74.6	30.8	34.5	74.3	26.5	34.5	74.2	21.3	27.7
92.2	36.7	41.5	92.0	32.2	41.0	91.6	25.6	33.3
98.4	38.6	44.1	98.2	34.3	43.8	97.9	28.1	36.7
116.4	44.0	50.7	116.2	38.2	48.7	116.0	30.8	40.2
118.9	45.9	52.8	119.1	40.4	51.6	118.0	32.2	42.0
119.2	...	...	119.2[b]	...	...	118.3[b]	...	...
147.1	...	55.6	146.8	...	54.8	146.5	...	45.0

[a] Fractions of doses recovered for Rats A, B, and C were, respectively, 0.837, 0.896, and 0.809. [b] Time at which implants were removed.

cretion rate diminishes with time. This decrease in excretion rate should parallel the decrease in absorption rate of the implant because of area changes which occur during absorption. When such a plot is made from the data presented in Table I of Latven and Welch (9), a decrease in excretion rate with time is observed. However, it cannot be determined from their data the values of the initial and final areas of the implants. Further, it should be possible to equate implant absorption rate to the urinary excretion rate of free and metabolized drug when implant surface area and fraction of the total dose recovered are considered and when absorption is solution rate limited.

An animal implanted with a drug pellet of a geometric form such that only small decreases in area occur with absorption may be likened to one given a continuous infusion of a drug when absorption from implant is solution rate limited. For the infused animal the relationship

$$Ri = Re/f \qquad \text{(Eq. 1)}$$

should apply after equilibrium is established, assuming drug removal processes are first order which is the usual case, where Ri is the constant rate of infusion, Re is the rate of urinary excretion, and f is the fraction of the total dose excreted in the urine in a time infinite in terms of the experiment. A similar relationship should hold in the case of implants after surface area corrections are made. Then the mean absorption rate per mean area, $\bar{R}/\bar{A}$, should equal the mean excretion rate per mean area, $\overline{Re}/\bar{A}$, divided by the fraction, f

$$\bar{R}/\bar{A} = (\overline{Re}/\bar{A})/f \qquad \text{(Eq. 2)}$$

assuming that no deposition of drug occurs at another site (*i.e.*, the kidney) (12, 13).

$$\bar{R}/\bar{A} \text{ per pellet} = \frac{(W_i - W_f)/t_i}{(A_i + A_f)/2} \qquad \text{(Eq. 3)}$$

$$\overline{Re}/\bar{A} \text{ per pellet} = \frac{(W_e/N)/t_e}{(A_i + A_f)/2} \qquad \text{(Eq. 4)}$$

where W_i and W_f are the initial and final mean pellet weights, t_i is the time of implantation, A_i and A_f are the initial and final areas of the implant, W_e is the weight of total drug recovered in the urine in time t_e, N is the number of pellets used, and t_e is the time of excretion.

TABLE III.—MATERIAL BALANCE: SHOWING THAT $\bar{R}/\bar{A} \cong (\overline{Re}/\bar{A})/f$

Rat	$\bar{R}/\bar{A}$ Gm./hr./cm.²	$(\overline{Re}/\bar{A})/f$ Gm./hr./cm.²
A	1.74×10^{-4}[a]	1.66×10^{-4}[b]
B	1.57×10^{-4}	1.47×10^{-4}
C	1.49×10^{-4}	1.40×10^{-4}

[a] $\bar{R}/\bar{A}$ per pellet $= \dfrac{(Wi - Wf)/ti}{(Ai + Af)/2} = \dfrac{(0.0508 - 0.0342)/119.2}{(0.864 + 0.740)/2} = 1.74 \times 10^{-4}$

[b] $(\overline{Re}/\bar{A})/f$ per pellet $= \dfrac{(We/N)/te}{(Ai + Af)/2}/f = \dfrac{(1/4 \times 0.0528)/118.9}{(0.864 + 0.740)/2}/0.837 = 1.66 \times 10^{-4}$

Table III shows the agreement between the experimental values of $\bar{R}/\bar{A}$ and $(\overline{Re}/\bar{A})/f$. Similar agreement was also observed when a different method of calculating $\overline{Re}/\bar{A}$ was used (13). The value of the $(\overline{Re}/\bar{A})/f$ is slightly smaller than that for $\bar{R}/\bar{A}$. This is expected since time in the beginning of the experiment is required for the establishment of equilibrium in drug absorption, distribution, metabolism, or excretion of drug; *i.e.*, t_e by this method is slightly larger than it should be.

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method described allows a plant to be tested in about six minutes, and the full requirements of chemicals and apparatus for one or two days' operations to be housed in a small carrying-case.

EXPERIMENTAL

About 2–4 Gm. of fresh plant material, preferably leaf and soft stems, is ground in a 3 in. unglazed porcelain mortar with a small amount of clean sand and sufficient chloroform to yield a thick slurry. Fine grinding to break down cell structure is important for rapid extraction. Ammoniacal chloroform (10 ml., *N*/20 with respect to ammonia) is added and the mixture stirred for about one minute before filtering the chloroform into a 5 × 1/2 in. test tube. Sufficient recovery of extract is obtained by pressing the material in the filter with a finger. Dilute sulfuric acid (2 *N*, 0.5 ml.) is added, the test tube shaken with a finger closing the end, and the phases allowed to separate (one minute or less is usually sufficient). The aqueous layer, or portion thereof, is removed with a dropper whose tip is fitted with a cotton wool plug for filtering and breaking emulsions. After removing the cotton wool and any chloroform in the dropper, two or three drops of the aqueous solution are placed in two 1 in. × 1/4 in. test tubes for testing with Mayers reagent or silicotungstic acid. The density of precipitate formed is assessed on a + to ++++ basis. Mortar, pestle, and filter funnel are wiped immediately with paper tissue but not washed after a negative test. Test tubes (5 × 1/2 in.) and droppers are emptied and re-used without washing after a negative test. After a positive result, mortar, pestle, and funnel are rinsed with chloroform and re-wiped, and test tube and dropper are rinsed with water from a wash-bottle. These precautions are sufficient to avoid contamination of a sample by the preceding one, while keeping the time required and usage of water at a minimum.

Fig. 1.—Alkaloid testing kit. At rear: Water wash bottle (100 ml.); chloroform containing *N*/20 ammonia (250 ml.); chloroform (250 ml.); 2 *N* sulphuric acid with calibrated dropper (100 ml.); Mayers reagent (50 ml.); silicotungstic acid solution (50 ml.); Center: Droppers with cotton wool filter plug (30); 2 in. funnel; 5 in. × 1/2 in. test tubes (24); 1 in. × 1/4 in. test tubes (several dozen). Front: Folded 11 cm. filter papers (24), 3 in. × 3/4 in. test tube calibrated to measure 10 ml.; 3 1/2 in. mortar; pestle; scissors; sand (250 ml. bottle).

The carrying case is illustrated in Fig. 1. All items are well embedded in balsa wood to prevent breakage or spillage under rough conditions. Polystyrene foam, although lighter in weight, is not satisfactory in place of balsa wood since it collapses wherever chloroform is spilled. Under field conditions, restocking is carried out each evening.

RESULTS

Results of the field test have been compared with those of a laboratory method which employs extraction of 20 Gm. dried milled plant with hot ethanol, removal of the ethanol, partitioning of the residue between ether and aqueous ammonia, and recovery of base from the ether with aqueous acid. The initial comparison was of results obtained in the field on fresh leaf material, with those obtained by the laboratory procedure on the same samples after drying and storing for up to three months. A limitation of the field method is that it misses all quaternary alkaloids, and the following comparisons are based only on the test for tertiary bases. The two methods gave identical results on 114 samples out of 147, 104 being negative, 4 weakly positive, and 6 strongly positive. The field test gave negative results for 14 species which were weakly positive by the laboratory method. The field test gave positive results (7 weak, 12 medium to strong) with 19 species for which the laboratory method gave only doubtful positives.

In view of the different condition of the samples, this comparison is useful only as evidence that the field method is a reliable screening procedure. Since our experience is that a weakly positive result (1+) in the laboratory test indicates that a plant is rarely worth further examination, the negative field result for 14 species in this category would not lead to any useful material being lost. The 12 species giving medium or strong field tests but weak laboratory tests may contain amines of very low molecular weight or suffer decomposition of alkaloid during drying of the plant material.

In order to compare the two methods more closely, a second group of 49 species was examined with both tests made from the same sample of fresh leaf material. Forty-four species gave the same result in both tests, 30 being negative, 10 strongly positive, and 4 weakly positive. Five species were strongly positive in the field test but only weakly positive by the laboratory method.

Our conclusion is that the field method with fresh material is a reliable screening procedure in so far as no species are missed which would have attracted further attention on the basis of the laboratory method. Since the field test is much simpler and can be performed more rapidly than the other, it may find application in circumstances other than that for which it was designed.

Gas Chromatographic Analysis of Acetylsalicylic Acid, Acetophenetidin, and Caffeine Mixture in Pharmaceutical Tablet Formulations

By ALLAN J. HOFFMAN and H. I. MITCHELL

THE WIDESPREAD use of APC mixtures suggested the possibility that the technique of gas chromatography might be useful as an analytical method. Although it was felt that the chemically dissimilar natures of the ingredients and the widely differing quantities of drug mixtures in tablet formulations might present a problem, a single-run type of analysis was attempted. The results are reported below.

EXPERIMENTAL

Equipment.—A linear programed-temperature gas chromatograph, F & M Scientific Corp. model 609, equipped with a flame ionization detector, was used for the experimental work. The detector signal was supplied to a model Y153 Minneapolis-Honeywell 1-mv. recorder, with a 1-second full scale response and a chart speed of 20 inches per hour. A Disc Chart Integrator model No. 201B (Disc Instrument, Inc., Santa Ana, California) accessory to the recorder was also used. For injection a 10-μl.-capacity Hamilton syringe was employed.

Materials.—Helium (Matheson Co.) was used as carrier gas. Haloport F 60–80 mesh (a dispersion polymer of a tetrafluoroethylene obtainable from F & M Scientific Corp., Avondale, Pa.) was coated with 2% by weight of Dow Corning 200 Fluid, and packed into a 6-ft. stainless-steel column, 4 mm. I.D., with the aid of a vibrating motor shaft. The chemical materials used in the investigation were purchased from commercial sources.

Operating Conditions.—The column temperature was programed, in all cases, from 75 to 200°. The heating rate found optimal was 9° per minute. The flow rate of helium was maintained at 50 ml./min. ±3% when the inlet pressure was kept at 50 psig. This constancy was essential for quantitative results. The sample injection port was maintained at 325°, and the detector block at 275° was employed for all experiments. Division of the peaks was performed vertically at the minima or by the inside tangent method, and peak areas were estimated by use of the automatic integrator. Two-microliter injections of chloroformic solutions were used throughout.

Quantitative Analysis.—Internal normalization, using correction factors for the conversion of peak areas to weight composition, was employed. Synthetic APC mixtures of the same percentage composition found in commercial tablets were prepared (see Table I). Correction factors were obtained by dividing the calculated weight per cent of each component of interest by its corresponding peak area per cent. Sample mixtures were then analyzed by multiplying the peak area of each component by the corresponding correction factor. The corrected peak areas were then normalized in the usual procedure. A sample of the procedure is given in Table I (top).

Tablet A consisted of a commercial APC tablet, uncoated, containing no other active ingredients, and of the composition indicated. The ground equivalent of four tablets was placed into a 100-ml. volumetric flask and diluted to the mark with chloroform. The mixture was then stirred magnetically for 1 hour and filtered through a No. 42 Whatman paper. Ten milliliters of the filtrate was evaporated to dryness in a current of dry air at about 40 to 45°. The residue was taken up in exactly 2 ml. of chloroform. Two microliters of this solution was injected to yield a chromatogram of the form shown in Fig. 1.

Tablet B consisted of a commercial APC tablet that also contained chlorpheniramine maleate. Results are shown in Table I (lower portion).

A commercial tablet, containing 8.1 mg. of codeine phosphate per tablet in addition to an APC mixture of the same weight per cent composition as Tablets A and B, was analyzed by this procedure. The chromatograms were in every way identical to those of the other tablets and to the synthetic mixture, indicating that codeine does not interfere under the conditions employed. Some quantitative results are shown in Table I under Tablet C.

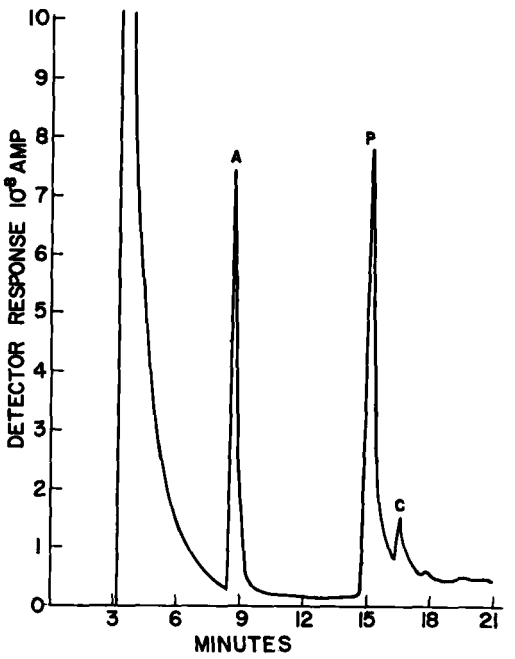

Fig. 1.—Typical gas chromatographic recording obtained for acetylsalicylic acid, acetophenetidin, and caffeine mixture with Dow Corning 200 fluid on Haloport F. Range 1000. Attenuation × 4.

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TABLE I.—PRECISION AND ACCURACY ANALYSIS OF A SYNTHETIC APC MIXTURE

	Component	Composition by Synthesis, % by Wt.	Run 1	Run 2	Run 3	Run 4	Mean	Av. Deviation from Mean, %	Av. Error, %
Synthetic mixture	Acetylsalicylic acid	53.52	53.31	55.06	54.33	54.75	54.36	±0.6	+1.6
	Acetophenetidin	38.34	38.36	37.12	37.74	37.96	37.79	−1.0	−1.4
	Caffeine	7.65	8.31	7.81	7.92	7.28	7.83	±3.6	+2.3
			Tablet Assays						
	Component	Labeled Composition, mg.	Run 1, mg.	Run 2, mg.	Run 3, mg.	Run 4, mg.	Run 5, mg.	Run 6, mg.	Mean, mg.
Tablet A	Acetylsalicylic acid	226.8	223.53	216.14	221.56	235.10	221.17	222.10	223.14
	Acetophenetidin	162.0	168.35	172.61	167.92	162.66	166.29	166.92	167.45
	Caffeine	32.4	32.4	32.4	31.72	31.34	33.70	32.10	32.28
Tablet B	Acetylsalicylic acid	226.8	224.64	217.04	226.36	227.70	225.23	...	224.19
	Acetophenetidin	162.0	161.78	170.21	161.48	160.00	162.16	...	163.11
	Caffeine	32.4	34.75	33.87	33.28	33.45	33.78	...	33.85
Tablet C	Acetylsalicylic	226.8	217.36	218.29	224.14	...	...	...	219.92
	Acetophenetidin	162.0	169.30	169.61	165.35	...	...	...	168.09
	Caffeine	32.4	33.98	32.77	31.17	...	...	...	32.64

SUMMARY AND DISCUSSION

GLC has been investigated with respect to its applicability to dosage forms containing APC mixture. It was felt that (*vis-a-vis* the N.F. column chromatographic technique) a method might be developed that would permit a greater number of replicate samples to be assayed, obviate the need for daily preparation of uniform columns, and release spectrophotometric facilities for other use.

This paper reports preliminary findings *via* a single-run type of assay. The results indicate that while the reliability of a single assay is less than in the official method, the means of the runs performed yielded results close to either labeled claim or composition by synthesis.

Efforts are being made to increase the precision of the technique and results will be reported in a future paper.

Communications

Possible Error in the Use of Polynomial Approximations in Urinary Excretion Rate Studies

Sir:

The utilization of urinary excretion data for the estimation of absorption rates has been employed in several reported studies (1–3). These papers indicate the method has certain advantages over one which bases its calculations on the concentration of the drug in blood plasma. Even though direct determination of plasma concentration of drug is recognized as a more accurate procedure, urinary excretion data are frequently employed since samples may be obtained at greater frequencies with a minimum of inconvenience to the subject, and drug concentration is such as to preclude assay difficulties.

Theoretical considerations of urinary excretion kinetics as related to absorption rate are reflected in the equation

$$dAe/dt = KfA_b$$

where dAe/dt is the rate of drug excretion; $K = 0.693/t_{1/2}$ where $t_{1/2}$ is the half-life of the drug in the body; f is the fraction of drug which is excreted in the urine unchanged; and A_b is the amount of drug in the body fluids. This equation is valid when the drug in the plasma is in equilibrium with other tissues and fluids of distribution. On the basis of this direct proportionality between excretion rate and the amount of drug in the body fluids, it is apparent that measurements of excretion rates plotted against time are far more indicative of rate of drug absorption than are plots of cumulative amounts of drug in the urine *vs.* time.

Excretion rates are defined as the instantaneous rate of change, at any given time, of cumulative

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Efforts are being made to increase the precision of the technique and results will be reported in a future paper.

Communications

Possible Error in the Use of Polynomial Approximations in Urinary Excretion Rate Studies

Sir:

The utilization of urinary excretion data for the estimation of absorption rates has been employed in several reported studies (1–3). These papers indicate the method has certain advantages over one which bases its calculations on the concentration of the drug in blood plasma. Even though direct determination of plasma concentration of drug is recognized as a more accurate procedure, urinary excretion data are frequently employed since samples may be obtained at greater frequencies with a minimum of inconvenience to the subject, and drug concentration is such as to preclude assay difficulties.

Theoretical considerations of urinary excretion kinetics as related to absorption rate are reflected in the equation

$$dAe/dt = KfA_b$$

where dAe/dt is the rate of drug excretion; $K = 0.693/t_{1/2}$ where $t_{1/2}$ is the half-life of the drug in the body; f is the fraction of drug which is excreted in the urine unchanged; and A_b is the amount of drug in the body fluids. This equation is valid when the drug in the plasma is in equilibrium with other tissues and fluids of distribution. On the basis of this direct proportionality between excretion rate and the amount of drug in the body fluids, it is apparent that measurements of excretion rates plotted against time are far more indicative of rate of drug absorption than are plots of cumulative amounts of drug in the urine *vs.* time.

Excretion rates are defined as the instantaneous rate of change, at any given time, of cumulative

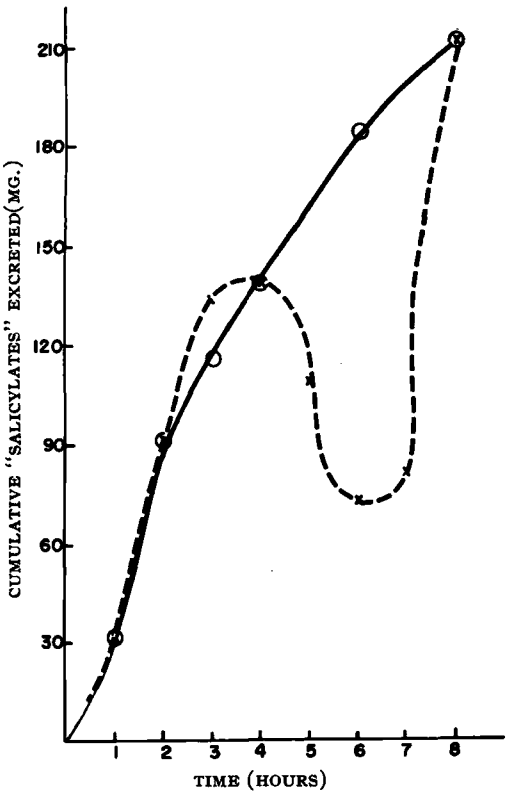

Fig. 1.—Comparison of actual and polynomial approximation curves. O, cumulative "salicylates" in urine in human subjects; X, polynomial approximations derived from 1, 2, 4, and 8 hour cumulative salicylate data.

amount of drug in the urine. Two basic methods may be used for their evaluation: graphical determination, or mathematical approximation of the Cumulative Amount *vs.* Time Curve with subsequent differentiation of the formula obtained.

The former method is tedious and requires a multiplicity of data for accurate determinations. The latter method, with the aid of a calculator, is to be favored both for rapidity and accuracy.

It has recently been proposed (2, 4, 5) that polynomial equations of fourth-order magnitude may be utilized to approximate the Cumulative Drug *vs.* Time Curve. The subsequent differentiation of the derived equations would directly yield the excretion rate at any time where the polynomial gives a good fit to the observed data.

The current literature in this area contains no reference to validation of reported data by a comparison of the instantaneous rates determined by polynomial approximation with either another approximation, or with graphical estimates. In addition, no graphical comparisons have been reported between experimental data and the polynomial approximation of these data.

Studies on excretion rates of salicylate currently being conducted in our laboratories indicate errors of rather large magnitude may be introduced in the mathematical treatment of data when using polynomial equations. The comparative plots in Fig. 1 indicate a possible degree of error which may be encountered in such approximations. The polynomial curve was derived from the actual cumulative salicylate levels of 30.5 mg. at the first hour, 90.3 mg. at the second, 138.9 mg. at the fourth, and 212.6 mg. at the eighth hour after ingestion. These data, obtained from one human subject, are typical of results obtained with other subjects in the study.

These results yielded the equation

$$x = -5.556t + 48.801t^2 - 13.766t^3 + 1.021t^4$$

where t is the time in hours and x equals the approximated cumulative milligrams of salicylate in urine.

In an effort to determine whether the range of the curve exercised any control on the accuracy of the approximation, a shorter plot covering the first 4 hours was calculated. The cumulative milligrams of salicylate at the third hour was 116.4 and this point, together with those for the first, second, and fourth hours, were utilized to derive

$$x = -28.43t + 88.83t^2 - 33.78t^3 + 3.88t^4$$

The fourth hour comparative curves in Fig. 2

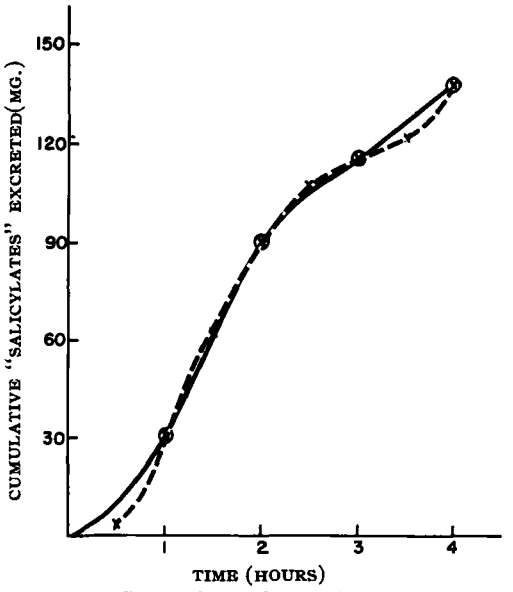

Fig. 2.—Comparison of actual and polynomial approximation curves. O, cumulative "salicylates" in urine in human subjects; X, polynomial approximations derived from 1, 2, 3, and 4 hour cumulative salicylate data.

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The Editor comments

POSTGRADUATE READING— THE HALLMARK OF A PROFESSIONAL

Some months ago a report came to our attention which struck us as reflecting a considerable degree of professional motivation in a particular group of pharmacy students. The report briefly and factually described a project which has been under way at Columbia University College of Pharmacy since late 1961.

Under the guidance of faculty advisor Dr. Gilbert Hite, the A.PH.A. Student Chapter decided to undertake a project which they aptly titled, *Postgraduate Reading is the Hallmark of a Professional.* A committee of three students then conducted a vigorous campaign using posters and displays, and sending a personal letter, together with a sample copy of THIS JOURNAL, to each student at the College. Their main goal was to interest the students in the monthly review articles, and various ideas were employed to publicize the review articles as well as the change in journal format, and to indicate the courses in which the journal articles would be most useful.

The purpose of the campaign was stated by the students as follows: "To stimulate postgraduate reading as a means of learning the new, recalling the useful, and dispossessing the old; and to stimulate interest in postgraduate studies." The committee also pointed out various secondary benefits, such as the building up of a useful personal library.

During the first year, 125 students (35 per cent) of the undergraduate student body subscribed to THIS JOURNAL. The campaign was renewed during the current year with even more spectacular results, the number of subscribers having increased to over 70 per cent of the undergraduate enrollment. Clifford Restler, the student committee chairman, states that the goal for next year is 100 per cent participation, and adds that the student chapters at other pharmacy schools should consider similar programs.

We wish to take this occasion to congratulate both the student chapter and the cooperative faculty at Columbia. The completely *voluntary and spontaneous* nature of this entire project is a real tribute to the professional attitude of the students and reflects their sincere dedication to develop themselves into knowledgeable pharmaceutical experts.

Edward G. Feldmann

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Review Article

Synthesis of Tetracycline Analogs

By G. C. BARRETT

NATURAL PRODUCTS possessing valuable physiological activity almost invariably have complicated structures; but simpler analogs representing part or parts of a natural product molecule may possess comparable activity, and the synthesis and testing of possible analogs has yielded useful new pharmaceuticals. This empirical approach to the synthesis of new drugs and antibiotics will eventually be replaced by more direct routes as soon as the mode of action of each family of compounds is fully understood. But while such knowledge is being slowly accumulated, useful advances have often accompanied the patterns of physiological activity shown by numerous analogs.

This article reviews the contributions of the study of analogs to the present understanding of the structural features essential for the characteristic activity of the tetracycline antibiotics. Large numbers of tetracycline analogs have been prepared by chemical modification of the natural products, and by modified fermentation techniques, and a comparative assessment of "tetracycline activity" in this series has revealed some of the basic structural requirements, variation of which may further yield simpler, highly active analogs. Physiologically active degradation products of the antibiotics are available by total synthesis, and other linearly fused tetracyclic, tricyclic, and bicyclic compounds have been prepared as potentially useful substitutes.

Received from the Department of Chemistry, University of Exeter, Devonshire, England.

The author thanks Dr. J. A. Barltrop, University of Oxford, for his introduction to and stimulating guidance in the field of tetracycline chemistry.

The objective of the present review may thus be fulfilled by a sequence of subsections describing the properties of the known tetracyclines and of the closely related compounds obtained from them; a critical discussion of the theory of the mode of action of the tetracyclines, and experimental facts relating to this theory; and the results of the synthesis and testing of analogs. Other reviews of the tetracyclines have appeared, stressing either their chemistry (1–4) or their general pharmacological properties and use (5, 6). General reviews are available (7, 8); that by Professor Shemyakin and his colleagues is a particularly complete account—both of the chemical and pharmacological aspects of the subject—of work published to mid-1960. The Russian treatise is to be published in English translation (9); the present review, therefore, aims at a complete literature coverage of the chemistry and pharmacological activity of the antibiotics and their analogs for the period 1960 to mid-1962. All known tetracycline analogs and reported pharmacological properties are listed in this review (Tables I and II), but the primary literature references quoted in (8) are not repeated here. However, the section describing *Totally Synthetic Tetracycline Analogs* aims at a complete coverage of the literature.

NATURALLY OCCURRING TETRACYCLINES

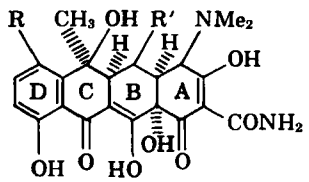
A strain of *Streptomyces*, uncataloged in 1948, was found to produce a remarkably effective antibiotic (10) which has since proved to be the first-discovered tetracycline. The pure active princi-

TABLE I.—TETRACYCLINES PRODUCED BY *Streptomycetes*

Name	Activity	References
Tetracycline	Highly active (8)	(8, 16, 17, 156)
7-Chlorotetracycline	Three to four times as active as tetracycline (8, 22, 60)	(8, 10, 11)
5-Hydroxytetracycline	Same activity as tetracycline (8, 22, 36, 60)	(8)
7-Bromotetracycline	Similar to chlortetracycline (8)	(19, 20, 21)
6-Demethyltetracycline	Same activity as tetracycline (8, 22, 60)	(22)
6-Demethyl-7-chlorotetracycline	Slightly less active than chlortetracycline (8, 22, 60)	(22)
5a,11a-(5,5a-?)[a] Dehydro-7-chlorotetracycline	Inactive (60). More than 50 times less active than tetracycline against *S. aureus* (8, 23)	(23)
2-Acetyl-2-decarboxamidotetracycline	Only 10% of the activity of tetracycline	(25)
2-Acetyl-2-decarboxamido-7-chlorotetracycline	Only 30% of the activity of tetracycline	(26)
2-Acetyl-2-decarboxamido-5-hydroxytetracycline	Only 10% of the activity of tetracycline	(26)

[a] Scott and Bedford (24) have suggested that the infrared spectrum of the dehydro-7-chlorotetracycline is more compatible with the 5,5a-location of the double bond.

ple was isolated as an orange-yellow hydrochloride (11); it was consequently named Aureomycin,[1] and its source was named *Streptomyces aureofaciens*. Degradative studies (12, 13) pointed to structural similarities with Terramycin,[2] a broad-spectrum antibiotic discovered in 1950 as a metabolite of *Streptomyces rimosus* (14). The brilliantly conducted study of the chemistry of Terramycin had progressed rapidly, and its structure and probable stereochemistry was published in 1953 (15); the name "tetracycline" was proposed (12) for a structure (I; R = R' = H) to which Aureomycin and Terramycin were simply related. Convenient to this nomen-

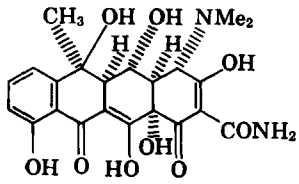


I

R = R' = H, tetracycline
R = Cl, R' = H, Aureomycin[1]
R = H, R' = OH, Terramycin[2]

clature was the later discovery that tetracycline itself is a highly active antibiotic which can be obtained by hydrogenolysis of chlortetracycline (16, 17), and is also produced by *S. aureofaciens* and by other *Streptomycetes* (18). Modifications to the environment in which the antibiotics are produced and the use of mutant *Streptomycetes* have since yielded a range of differently substituted tetracyclines. The members of this family available by biosynthesis are listed in Table I.

Results of the X-ray structural analysis (28) of oxytetracycline suggest that the molecule should be depicted as II.



II

Chemical evidence has been accumulated in support of both the α-orientation II (51, 157) and of the β-orientation I (15) of the dimethylamino-substituent at the 4-position in oxytetracycline; but the ease of epimerisation at this position must be responsible for these conflicting results (51), and it might be wisest now to picture naturally occurring 4- and 5-substituted tetracyclines as possessing the α-orientation at these positions, as shown in II. Recent papers (24, 29) are, indeed, adopting the relative stereochemistry indicated by the X-ray method. Independent X-ray studies (27, 163) have shown further that chlortetracycline and oxytetracycline possess the same configurations at all common asymmetric centers, and it is probable that all other naturally occurring tetracyclines may be assigned to this same configurational family.

CHEMICAL MODIFICATION OF NATURALLY OCCURRING TETRACYCLINES

Extension of the available totally synthetic routes to yield new tetracyclines cannot be an economic proposition, since the natural products are now relatively cheap and may be converted into a range of differently substituted tetracyclines by simple procedures. The variety of functional groupings, while conferring on the

[1] Subsequently the trade name of Lederle Laboratories for chlortetracycline hydrochloride.
[2] Subsequently the trade name of Chas. Pfizer and Co. for oxytetracycline hydrochloride.

molecule a bewildering complexity, makes it possible to envisage a host of small, feasible modifications; some modifications have been performed and, in general, the resulting compounds retain antibiotic activity if the natural configuration of the intact framework of the molecule is unaltered. Epimerisation at positions 4 and 5a results in almost complete loss of activity,[3] and the natural stereochemistry of the molecule is thus shown to be an essential prerequisite of physiological activity (105). Even though the inversion at C_6 which occurs (29) during the removal of the 6-hydroxy group by catalytic hydrogenolysis does not significantly reduce the antibiotic activity of the molecule, 6-deoxytetracyclines with the natural configuration of the 6-methyl group are appreciably more active than their C_6-epimers (29).

Methods are available for the selective removal and for the alteration of some existing substituents, and for the introduction of new functional groupings. New tetracyclines thus obtained from the natural products are listed in Table II, and their reported activities are quoted. A place is found in the Table for any particular modified tetracycline by checking its formula through the following sequence:

(*A*) 6-Deoxytetracyclines
  (*a*) 6-Deoxytetracyclines (excepting 5a,-6-anhydrotetracyclines)
  (*b*) 6-Demethyl-6-deoxytetracyclines
  (*c*) 6-Methylene-6-demethyl-6-deoxytetracyclines
  (*d*) 5a,6-Anhydrotetracyclines
  (*e*) Dedimethylamino-5a,6-anhydrotetracyclines
(*B*) 2-Carboxamide-modified tetracyclines
(*C*) Dimethylamino-modified tetracyclines
(*D*) Dedimethylamino-tetracyclines
(*E*) O-Acylated- and O-alkylated-tetracyclines
(*F*) Other modified tetracyclines

Comparative figures are available for many of the more active naturally occurring and chemically modified tetracyclines, and these figures are listed in Table III. The commonly used assay methods (57, 58) yield some inconsistent results, and activities are therefore quoted in Table II in qualitative terms, either in the words used in the reference cited or as implied by the figures in Table III.

Although a useful variety of transformations has been achieved in this series, it should be emphasized that the methods now available lead only to certain classes of analogs. Thus, D-ring substituents cannot be introduced by electrophilic substitution reactions into the parent tetracyclines, but may be introduced into more stable 6-deoxy- and 5a,6-anhydrotetracyclines.

Many analogs are thus potentially accessible only by total synthesis; but the development of methods which can augment the range of modified tetracyclines may now be expected. The new methods may involve ever more circuitous routes, and the general pattern is likely to involve the conversion of the natural products into their more stable anhydro-derivatives, followed by specific substitution and reconversion into the substituted tetracycline by oxygenation. The 6-hydroxy-group can be introduced into anhydroaureomycin by microbiological agents (59), or *via* the 6-hydroperoxy-compound (obtained by bubbling oxygen through an irradiated benzene solution of the anhydro-compound) (24), and these procedures, which yield the tetracycline with the natural C_6 configuration, are likely to be widely used. Oxygenation of certain 12a-deoxytetracyclines is also a simple matter by a microbiological method (*Circularia lunata*) (62, 63), by means of perbenzoic acid (64) [not applicable to 12a-deoxytetracycline (65, 51)], or with oxygen in the presence of sodium nitrite (61, 63) or in the presence of a noble metal (66). Other inorganic oxidizing agents can be used for 12a-hydroxylation (150).

The range of modified tetracyclines listed in Table II includes derivatives (for example, the halogen-substituted and diazonium modifications) suitable for further elaboration into antibiotics with novel substituents; particularly, the 6-methylenetetracyclines offer an entry into a new family of 6-substituted 6-deoxytetracyclines likely to possess high activity.

MODE OF ACTION OF THE TETRACYCLINES

The tetracycline molecule clearly depends more for useful activity upon the presence of the sequence of oxygen functions, from the phenolic hydroxyl at C_{10} and the groups at C_{11} and C_{12} through to the ring A 1,3-diketone grouping, than upon the presence of substituents at carbons 4–9. Other essential structural requirements also may be deduced from the relative activities of the various classes of modified tetracyclines; first, that the 2-carboxamide substituent also seems to have an essential role, since its replacement by a cyano- or acetyl-grouping greatly reduces antibiotic activity in this series. 2-Carboxamide-N-mono-substituted tetracyclines retain full activity, and this may indicate that the —CO—NH— grouping at the 2-position is a minimum requirement for activity. This conclusion suggests that the 2-substituent should be capable of forming the chelated structure III

[3] 4-Epi-tetracyclines are active *in vivo* but are almost devoid of activity against the standard assay organisms; conversion into the natural isomer might occur in the tissues of the laboratory animals, resulting in the observed *in vivo* activity.

TABLE II.—SUBSTITUENT-MODIFIED TETRACYCLINES

-tetracycline	Activity	Refs.
-6-Deoxytetracyclines		
6-Deoxytetracycline	Slightly less active than tetracycline	(29)
6-epi-methyl-	Half as active as tetracycline (29); similar activity to tetracycline (8).	(8, 30, 38)
5-Hydroxy-	More active than oxytetracycline	(29)
6-Epi-methyl-5-hydroxy-	Less than half the activity of oxytetracycline	(8, 30, 38)
4-Epi-	.....	(38)
4-Epi-5-hydroxy-	.....	(38)
6-Epi-methyl-dedimethylamino-	.....	(8, 30)
6-Epi-methyl-7-bromo-	More active than tetracycline	(31, 32)
6-Epi-methyl-7-iodo-	Less active than tetracycline	(31, 32)
6-Epi-methyl-7-^{131}iodo-	.....	(32, 33)
6-Epi-methyl-7-nitro-	Half of the activity of chlortetracycline	(31, 159)
6-Epi-methyl-9-nitro-	Inactive	(31, 159)
6-Epi-methyl-9-amino-	One-tenth of the activity of chlortetracycline	(31, 159)
6-Epi-methyl-9-amino-7-bromo-	Thirty-five per cent of the activity of chlortetracycline	(31)
6-Epi-methyl-9-amino-7-nitro-	Forty per cent of the activity of chlortetracycline	(31)
6-Epi-methyl-9-acetamido-	Half as active as the 9-amino-compound	(159)
6-Epi-methyl-9-diazonium-	Weakly active	(32)
6-Epi-methyl-9-azido-	Weakly active	(32)
6-Epi-methyl-9-nitro-5-hydroxy-	Inactive	(34)
6-Epi-methyl-9-ethoxythiocarbonylthio-	.....	(32)
11a-Fluoro-5-hydroxy-	.....	(37)
11a-Fluoro-6,12-oxido-	.....	(37)
11a-Fluoro-6,12-oxido-5-hydroxy-	.....	(37)
11a-Fluoro-6,12-oxido-dedimethylamino-	.....	(37)
11a-Fluoro-6,12-oxido-5-hydroxy-dedimethylamino-	.....	(37)
11a-Chloro-6,12-oxido-	.....	(36)
11a-Chloro-6,12-oxido-5-hydroxy-	.....	(36)
7-Chloro-6-hydroperoxy-5a,11a-(5,5a-?) dehydro-	.....	(24)
-6-Demethyl-6-deoxytetracyclines		
6-Demethyl-6-deoxytetracycline	One to two times as active as tetracycline (see Table III)	(8, 30, 39)
4-Epi-	.....	(39)
6-Benzylthiomethyl-	.....	(29)
7-^{3}H-	.....	(32)
7-Chloro-	Three times as active as tetracycline	(32)
7-Bromo-	Two times as active as tetracycline	(32, 34)
7-Iodo-	Same activity as tetracycline	(31, 32)
7-Nitro-	Five to ten times as active as tetracycline (see Table III)	(31, 159)
7-Amino-	Half to two times as active as tetracycline (see Table III)	(31, 159)
7-Formamido-	Same activity as the 7-amino-compound	(159)
7-Diazonium-	Twenty per cent of the activity of tetracycline	(32)
7-Azido-	1$^1/_2$ times as active as tetracycline	(32)
7-Ethoxythiocarbonylthio-	Fifty per cent of the activity of tetracycline	(32)
7-Bromo-9-nitro-	Only slight activity	(34)
7,11a-Dibromo-	.....	(34)
9-Nitro-	Inactive (31); nearly half as active as tetracycline (34)	(31, 159)
9-Amino-	1$^1/_2$ times as active as tetracycline (32)	(31, 32, 159)
9-Formamido-	More active than the 9-amino-compound	(159)
9-Azido-	Same activity as tetracycline	(32)
9-Diazonium-	Slight activity	(32)
9-Ethoxythiocarbonylthio-	Slight activity	(32)
11a-Fluoro-	.....	(37)
11a-Fluoro-dedimethylamino-	.....	(37)
11a-Fluoro-6,12-oxido-	.....	(37)
11a-Chloro-	.....	(36)
11a-Chloro-5-hydroxy-	.....	(36)
11a-Bromo-	.....	(32, 34)
O^{12a}-Formyl-	.....	(35)
12a-Deoxy-	.....	(35)

TABLE II.—*Continued*

-tetracycline	Activity	Refs.
12a-Deoxy-dedimethylamino-	.....	(35)
4a,12a-Anhydro-	.....	(35)
-6-Methylene-6-demethyl-6-deoxytetracyclines		
6-Methylene-6-demethyl-6-deoxytetracycline	Same activity as tetracycline	(36)
7-Chloro-	.....	(36)
5-Hydroxy-	Two times as active as oxytetracycline	(36)
7-Bromo-5-hydroxy-	.....	(36)
7-Iodo-5-hydroxy-	.....	(36)
7-Chloro-5-hydroxy-	Six times as active as oxytetracycline	(36)
11a-Fluoro-	.....	(36)
11a-Chloro-	.....	(36)
11a-Chloro-5-hydroxy-	.....	(36)
7,11a-Dichloro-5-hydroxy-	.....	(36)
-5a,6-Anhydrotetracyclines		
5a,6-Anhydrotetracycline	Active against a number of gram-positive and gram-negative, and acid-fast bacteria, also against *actinomycetes*. More active than tetracycline Against *T. vaginalis in vivo* (8). Slight activity (60)	(8, 40)
4-Epi-	.....	(8)
5-Hydroxy-	.....	(8)
7-Chloro-	Active against a number of gram-positive, and gram-negative, and acid-fast bacteria; active against a number of *Actinomycetes* in concentration $\leqslant 1\gamma$/ml. More active than tetracycline against *T. vaginalis in vivo* (8); 5% of the activity of chlortetracycline	(8, 41)
7-Chloro-6-demethyl-	.....	(8)
7-Bromo-	.....	(8)
7-Chloro-9-benzyl-	Active against *T. vaginalis* in concentration 50 γ/ml.	(8)
7-Chloro-6-demethyl-12a-deoxy-	.....	(42)
9-*tert*-Butyl-	Active against *T. vaginalis in vivo* (1.6 γ/ml.) and more active *in vivo* than tetracycline (8)	(8, 158)
9-*tert*-Butyl-7-chloro-	Active against *T. vaginalis in vitro* (0.8 γ/ml.), more active than tetracycline *in vivo* (8)	(8, 158)
9-*tert*-Amyl-5-hydroxy-	.....	(8, 158)
5-Acetoxy-O^{12a}-acetyl-	Active against a number of microorganisms, including several resistant to tetracycline	(8)
12a-Deoxy-	Active against certain tetracycline-resistant bacteria (42)	(35, 42, 51)
12a-Deoxy-6-demethyl-	.....	(42)
4a,12a-Anhydro-	.....	(8)
7,8-Dihydro-12α-hydroxy-12-deoxo-	Inactive	(60)
7,8-Dihydro-12β-hydroxy-12-deoxo-	Inactive	(60)
7,8-Dihydro-12-deoxo-	.....	(8, 30, 43)
Benzaldehyde derivative of 7,8-dihydro-12-deoxo-	Antibacterial	(43)
Salicylaldehyde derivative of 7,8-dihydro-12-deoxo-	Antibacterial	(43)
p-Nitrobenzaldehyde derivative of 7,8-dihydro-12-deoxo-	Antibacterial	(43)
5-Nitrofurfuraldehyde derivative of 7,8-dihydro-12-deoxo-	Antibacterial	(43)
2-Cyano-2-decarboxamido-5-hydroxy-O^{10}-benzenesulfonyl-	.....	(8)
N^2-*tert*-Butyl	Active against a number of gram-positive, gram-negative, and acid-fast bacteria	(8, 131)
N^2-*tert*-Butyl-7-chloro-	Active against a number of gram-positive, gram-negative, and acid-fast bacteria; also several other microorganisms	(8)
N^2-*tert*-Butyl-9-*tert*-butyl-	Active against a number of microorganisms	(8)
N^2-*tert*-Butyl-9-*tert*-butyl-7-chloro-	Active against *T. vaginalis in vitro* (0.8 γ/ml.)	(8)
N^2-*tert*-Amyl-5-hydroxy-	.....	(8)
N^2-Piperidinomethyl-	.....	(8)
N^2-(Dimethylbenzyl)methyl-7-chloro-	.....	(8)
N^2-Acetonyl-5-hydroxy-	.....	(8)

TABLE II.—*Continued*

-tetracycline	Activity	Refs.
N^2-Acetonyl-5-acetoxy-O^{12a}-acetyl-	.....	(8)
N^2-(9-Xanthyl)-	.....	(8, 44)
Dedimethylamino-5a,6-anhydrotetracyclines		
Dedimethylamino-5a,6-anhydrotetracycline	.....	(8)
5-Hydroxy-	Active against a number of micro-organisms, including several resistant to tetracycline	(8)
5-Hydroxy-12a-epi-	Active	(8)
5-Hydroxy-12a-deoxy-	Active	(8)
5-Hydroxy-12a-deoxy-12a-epi-methyl-	Active	(8)
7-Chloro-	Active against a number of microorganisms, including several resistant to tetracycline	(8)
12a-Deoxy-	.....	(8, 50, 51)
12a-Deoxy-7-chloro-	.....	(8)
12a-Deoxy-7-chloro-6-demethyl-	.....	(8)
4a,12a-Anhydro-	.....	(8)
4a,12a-Anhydro-7-chloro-	.....	(8)
O^{10}-Methyl-	Active	(8)
O^{10}-Methyl-9-bromo-	.....	(103)
O^{10}-Methyl-12a-epi-	.....	(8)
O^{10}-Methyl-12a-deoxy-	.....	(8)
$O^{10,11}$-Dimethyl-	.....	(8)
$O^{10,11}$-Dimethyl-7-chloro-12a-deoxy-	.....	(8)
N^2-Acetonyl-	.....	(8)
N^2-Acetonyl-5-hydroxy-	.....	(8)
N^2-Acetonyl-7-chloro-	.....	(8)
N^2-Acetonyl-5-hydroxy-12a-epi-	.....	(8)
N^2-Acetonyl-5-hydroxy-12a-deoxy-	.....	(8)
N^2-Acetonyl-5-hydroxy-12a-deoxy-12a-epi-methyl-	.....	(8)
9-Chloro-	.....	(50)
9-Bromo-	.....	(50)
9,12a-Dibromo-12a-deoxy-	.....	(50)
(x)-Bromo-7-chloro-	.....	(50)
2-Carboxamide-modified tetracyclines		
2-Cyano-2-decarboxamido-	Less than 5% of the activity of tetracycline (8) Slight activity (60)	(8)
2-Cyano-2-decarboxamido-7-chloro-	.....	(8)
2-Cyano-2-decarboxamido-5-hydroxy-	.....	(8)
2-Cyano-2-decarboxamido-7-bromo-	.....	(8)
2-Cyano-2-decarboxamido-4-epi-7-chloro-	.....	(8)
2-Cyano-2-decarboxamido-O^{10}-benzenesulfonyl	.....	(8)
2-Cyano-2-decarboxamido-4-epi-O^{10}-benzenesulfonyl-	.....	(8)
2-Cyano-2-decarboxamido-5-hydroxy-O^{10}-benzenesulfonyl-	.....	(8)
N^2-Aminomethyl-	.....	(8)
N^2-Diethylaminomethyl-	.....	(8)
N^2-Dibenzylaminomethyl-	.....	(8)
N^2-(β-Hydroxyethyl)aminomethyl-	.....	(8)
N^2-Di(β-hydroxyethyl)aminomethyl-	.....	(8)
N^2-Di(β-diethylaminoethyl)aminomethyl-	.....	(8)
N^2-Pyrrolidinomethyl- ("Reverin")	Same activity as tetracycline against a wide range of gram-positive and gram-negative organisms	(8)
N^2-Pyrrolidinomethyl-5-hydroxy-	.....	(8)
N^2-Piperidinomethyl-	.....	(8)
N^2-Piperidinomethyl-4-epi-	.....	(8)
N^2-Piperidinomethyl-5-hydroxy-	.....	(8)
N^2-Morpholinomethyl-	Highly active	(8)
N^2-Morpholinomethyl-7-chloro-	.....	(8)
N^2-Morpholinomethyl-5-hydroxy-	.....	(8)
N^2-Morpholinomethyl-methiodide	Highly active	(8)
N^2-{N'-(β-Hydroxyethyl)}-piperazinomethyl-	.....	(8)
N^2-(N'-Methyl)piperazinomethyl-7-chloro-	.....	(8, 45)
N^2-(9-Xanthyl)-	.....	(8, 44)
N^2-(9-Xanthyl)-7-chloro-	.....	(44)
N^2-(9-Xanthyl)-7-bromo-	.....	(44)
N^2-(9-Xanthyl)-5-hydroxy-	.....	(44)
Dimethylamino-modified tetracyclines		
4-Epi-	Seven to 50 times less active than tetra-	(8, 46, 47)

TABLE II.—*Continued*

-tetracycline	Activity	Refs.
	cycline; active *in vivo* (8); slight activity (60)	
4-Epi-5-hydroxy-	Twenty-five times less active than tetracycline against *S. aureus;* active *in vivo* (8, 22)	(8, 22)
4-Epi-7-chloro-	Six times less active than tetracycline against *S. aureus;* active *in vivo* (8, 22)	(8, 22)
4-Epi-7-bromo-	Twenty times less active than 7-bromotetracycline against *S. aureus;* active *in vivo*	(8)
4-Epi-6-demethyl-	Eight times less active than tetracycline against *S. aureus* (8, 22)	(8, 22)
4-Epi-6-demethyl-7-chloro-	Four times less active than tetracycline against *S. aureus* (8, 22)	(8, 22)
4-Epi-12a-deoxy-	.....	(51)
Methiodide	Slight activity (8, 60)	(8, 48, 155)
4-Epi-methiodide	.....	(8, 48)
7-Chloro- methiodide	Slight activity	(8, 155)
12a-Deoxy- methiodide	.....	(35)
Dedimethylamino-tetracyclines		
Dedimethylaminotetracycline	Highly active against a variety of microorganisms (8); 15% of the activity of tetracycline (60)	(8, 155)
7-Chloro-	Highly active against a large number of microorganisms	(8)
5-Hydroxy-	Highly active against a number of microorganisms	(8)
7-Bromo-	Active	(8)
12a-Deoxy-	.....	(8, 13, 50, 51)
12a-Deoxy-7-chloro-	.....	(8)
12a-Deoxy-7-chloro-6-demethyl-	.....	(8)
12a-Deoxy-12a-epi-methyl-5-hydroxy-	.....	(8)
4,12a-Anhydro-	.....	(35, 50, 51)
O^4-Acetyl- (or O^9-acetyl-)	.....	(53)
11a-Bromo-	.....	(50)
11a,12a-Dibromo-12a-deoxy-	.....	(50)
12a-Bromo-12a-deoxy-	.....	(50, 52)
12a-epi-5-hydroxy-	.....	(138)
O-Acylated- and O-alkylated-tetracyclines		
O^{12a}-Formyl-	.....	(35, 49)
O^{12a}-Acetyl-	Highly active	(8)
O^{12a}-Acetyl-7-chloro-	Highly active	(8)
O^{12a}-Acetyl-5-hydroxy-	Highly active against a large number of microorganisms	(8)
O^{12a}-Acetyl-5-acetoxy-	Active	(8)
O^{10}-Acetyl-5-hydroxy-(?)	.....	(35)
$O^{10,12a}$-Diacetyl-5-hydroxy-	.....	(35)
O^{10}-Propionyl-5-hydroxy-(?)	.....	(35)
O^{12a}-Propionyl-5-hydroxy-	Highly active	(8)
$O^{10,12a}$-Dipropionyl-5-hydroxy-	.....	(35)
O^{12a}-Palmityl-	.....	(8)
O^{12a}-Phenylcarbamyl-	Antibacterial	(35, 153)
O^{12a}-Phenylcarbamyl-5-hydroxy-	.....	(35, 153)
O^{12a}-(*p*-Methoxyphenyl)carbamyl)-	.....	(153)
$O^{3,12}$-Dimethyl-5-hydroxy-	.....	(8)
Other modified tetracyclines		
7-^{3}H-	.....	(145)
7-^{3}H-6-demethyl-	.....	(31)
7-Chloro- -^{14}C	.....	(88)
7-^{36}Chloro-	.....	(21, 146)
7-^{36}-Chloro-5a,11a-(5,5a-?)dehydro-	.....	(147)
5a-Epi-	Less than 2% of the activity of tetracycline against *S. aureus* (8, 23, 60)	(8, 23, 46, 55)
12a-Deoxy-	Inactive (60); 2% of the activity of tetracycline against *S. aureus* (51) Appreciably active (35, 56).	(8, 35, 51, 56, 153, 154)
12a-Deoxy-5-hydroxy-(?)	.....	(35)
4a,12a-Anhydro-	Slight activity (35)	(35, 50, 51, 153)
9-Pyrrolidinomethyl-	.....	(54)

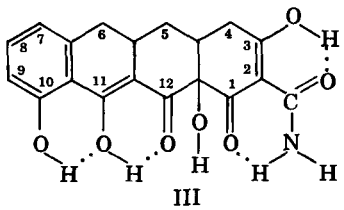
involving the oxygen functions at positions 1 and 3. Second, the removal of the 12a-hydroxyl group deprives tetracycline of its activity, but O^{12a}-esters are highly active (49); the presence of a substituent larger than hydrogen may be the simple requirement at this position. Thus, replacement of the 12a-hydroxyl group of dedimethylamino-5-hydroxy-5a,6-anhydrotetracycline by methyl with simultaneous inversion of configuration yields an active antibiotic, though the very different antibacterial spectrum of 5a,6-anhydrotetracyclines (suggesting that a different mode of antibacterial action applies to this class (130, 131)) reduces the validity of this comparison. The presence of a 9-nitro-substituent greatly reduces antibiotic activity, whereas 7-chloro-tetracyclines are invariably more active than their unsubstituted analogs. The phe-

TABLE III.—RELATIVE ACTIVITIES OF SUBSTITUENT-MODIFIED TETRACYCLINES

-tetracycline	Ref:	(31)	(26)	(60)	(60)	(36)	(32)	(22)	(29)	(34)
	Assay:	(58)	(57)	(58)[a]	(58)	(57)	(58)	[b]	(57)	(57)
Tetracycline			100	100	100	1000	100	25		
5-Hydroxy-				80	100	1000		24	1000	
7-Chloro-		100		350				100		
6-Demethyl-				100	95			24		
7-Chloro-6-demethyl-				300				75		
7-Chloro-5a,11a-(5,5a-?) dehydro-				1						
2-Acetyl-2-decarboxamido-			10							
2-Acetyl-2-decarboxamido-5-hydroxy-			10							
2-Acetyl-2-decarboxamido-7-chloro-			30							
6-Deoxy-									700	
6-Deoxy-6-epi-methyl-		18		70	70		70		500	
6-Deoxy-5-hydroxy-									1400	
6-Deoxy-6-epi-methyl-5-hydroxy-				50	36				400	
7-Bromo-6-deoxy-6-epi-methyl-		30					140			
7-Iodo-6-deoxy-6-epi-methyl-		14					60			
7-Nitro-6-deoxy-6-epi-methyl-		60								
9-Nitro-6-deoxy-6-epi-methyl-		<1								
9-Amino-6-deoxy-6-epi-methyl-		14					60			
9-Amino-7-bromo-6-deoxy-6-epi-methyl-		35								
9-Amino-7-nitro-6-deoxy-6-epi-methyl-		41								
9-Diazonium-6-deoxy-6-epi-methyl-							10			
9-Azido-6-deoxy-6-epi-methyl-							10			
9-Nitro-6-deoxy-6-epi-methyl-5-hydroxy-										<10
6-Demethyl-6-deoxy-		40		200	170		160		900	900
7-Chloro-6-demethyl-6-deoxy-							300			
7-Bromo-6-demethyl-6-deoxy-		60					200			1300
7-Iodo-6-demethyl-6-deoxy-		30					120			
7-Nitro-6-demethyl-6-deoxy-		160								4600
7-Amino-6-demethyl-6-deoxy-		21					40			975
7-Diazonium-6-demethyl-6-deoxy-							20			
7-Azido-6-demethyl-6-deoxy-							150			
7-Ethoxythiocarbonylthio-6-demethyl-6-deoxy-							50			
7-Bromo-9-nitro-6-demethyl-6-deoxy-										25
9-Nitro-6-demethyl-6-deoxy-		3								200
9-Amino-6-demethyl-6-deoxy-		40					160			760
9-Azido-6-demethyl-6-deoxy-							90			
9-Diazonium-6-demethyl-6-deoxy-							17			
9-Ethoxythiocarbonylthio-6-demethyl-6-deoxy-							10			
6-Methylene-6-demethyl-6-deoxy-						1200				
6-Methylene-6-demethyl-6-deoxy-5-hydroxy-						2300				
6-Methylene-6-demethyl-6-deoxy						6300				
6-Deoxy-7-chloro-5-hydroxy-5a,6-anhydro-					6					
2-Cyano-2-decarboxamido-					1					
4-Epi-					6			1.6		
4-Epi-7-chloro-								4.2		
4-Epi-5-hydroxy-								1.1		
4-Epi-6-demethyl-								3		
4-Epi-7-chloro-6-demethyl-								7		
Methiodide				1						
Dedimethylamino-				15						
5a-Epi-				1						
12a-Deoxy-				1						

[a] Pelcak and Dornbush's method (58) was modified for this set of results by the addition of 10% normal horse serum to the test medium. This modification yields results more closely paralleling those of *in vivo* tests. [b] *In vitro* against *S. aureus*.

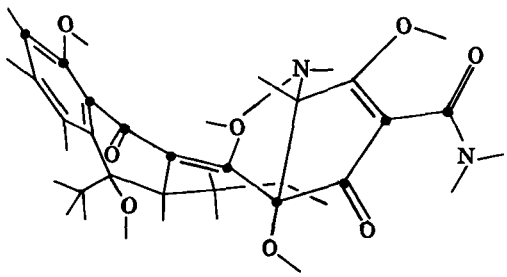
nolic hydroxyl group at position 10 is assumed to have an important role (*vide infra*), and its effective performance may be impaired by hydrogen-bonding with the nitro-group (34). The opposite effect of the 7-chloro-substituent is consistent with its capability (67) of electron-release to the para-position.



III

The deduction (138) that the 11 and 12 oxygen functions, augmented by the effect of the phenolic hydroxyl at C_{10}, are primarily responsible for holding metal ions with which tetracyclines form strong chelates (68–70) does not satisfactorily explain the function of the A-ring substituents, if the antibiotic activity of the tetracyclines is accepted to be due to the disturbance of certain enzyme systems by chelation of vital trace metals (68). Ferric ions are strongly held by the tetracyclines (68, 69); also by 8-hydroxyquinoline, which is a potent antibacterial (71–73). The tetracyclines may inhibit protein synthesis by chelation of essential metals, resulting in the production of unstable, small molecular weight RNA (74), but no experimental evidence is yet available in support of this theory. It is known, however, that chlortetracycline inhibits the action of an aromatic nitro-reductase by combining with manganese ions essential to the function of this enzyme (75); also that oxidative phosphorylation in liver mitochondria preparations, inhibited by the presence of chlortetracycline (76, 77), again proceeds normally when an excess of magnesium ions is added to the nutrient medium (77). Significantly, it has been found that the antibacterial action of chlortetracycline and of oxytetracycline is inhibited in the presence of magnesium ions (78). The conclusion, drawn from studies of *E. coli* extracts, that chlortetracycline exerts its antibiotic action by inhibiting electron-transport in sensitive organisms (79) takes account of the fact that the electron-transport enzymes and nitro-reductases of *Neurospora crassa* (80) and of *E. coli* (81, 82) are known to be metalloflavins. Chlortetracycline-sensitive nitro-reductase concentrates contain easily dissociable flavin, whereas the flavin is firmly bound in resistant species (83); the point of inhibition by the antibiotic is concluded to be the stage of reoxidation of conjugated flavoproteins (79).

It has been pointed out that epimerisation at positions 4 and 5a results in almost complete loss of antibiotic activity, and that the 6-epimer is significantly less active. As can be visualized from the scale drawing IV, the spatial relationships between parts of the molecule must be affected by epimerisation, but it is not obvious that the consequent changes in the shape of the molecule would limit the chelating ability of the sequence of oxygen functions involved—though interaction between the 4-dimethylamino-group and the oxygen functions at carbons 10, 11, and 12 is a distinct possibility in 4-epi-tetracyclines. Unless the varying antibiotic activities shown by modified tetracyclines can be directly related to their ability to form more or less strong metal chelates, then other factors must also be essential to the activity of this group of antibiotics.



IV

BIOSYNTHESIS

An exact understanding of the stepwise synthesis of tetracyclines *in vivo* can suggest ways by which new tetracyclines may be obtained from normal and mutant *Streptomycetes*, utilizing nutrient media in which new substrates replace the "building blocks" essential to the normal biosynthetic route.

The production of 7-chlorotetracycline by *S. aureofaciens* is dependent upon the presence of ionic chlorine in the nutrient medium, but the total yield of tetracyclines produced under conditions of low halide concentration is unchanged since tetracycline itself then forms in increased amount (21). Thiocyanate, added to the usual *S. aureofaciens* nutrient medium, also inhibits 7-chlorotetracycline synthesis but the total yield of tetracyclines is again unchanged (146, 21). The same organism can be induced to synthesize 6-demethyl-7-chlorotetracycline in addition to 7-chlorotetracycline by contamination of the nutrient medium with sulfonamides (84); the proportion of the 6-demethyl compound formed under these conditions can be made smaller by the addition of methionine to the medium. Halogenation and methylation are thus shown to be

late steps in the biosynthetic sequence. Mutant strains of the tetracycline-producing organisms can produce only modifications of the normal product of their earlier generations in some cases because they cannot complete the biosynthetic route; thus, the 7-chloro-5a,11a-(5,5a-?)dehydrotetracycline produced by *S. aureofaciens* "S 1308" (23, 59) is converted into 7-chlorotetracycline by the normal strain of the organism (59), also by other mutants (146). The 6-demethyl- and the 2-acetyl-2-decarboxamido-tetracyclines listed in Table I are also produced by mutant *Streptomycetes*.

The "head-to-tail" linkage of acetate units (86), predicted by Robinson (85), has been demonstrated to be the route by which the skeleton is largely built up, the 6-methyl substituent and the dimethylamino methyl groups being derived from methionine (87). Glutamic acid is probably the basis of the larger part of ring A(87).

Incorporation of isotopically-labeled acetic acid (both carboxyl-^{14}C- and methyl-^{14}C- labeled forms) (87, 88), glycine-2-^{14}C (88), and methionine-$^{14}CH_3$ (87) has been separately demonstrated. The dimethylamino group in chloro-

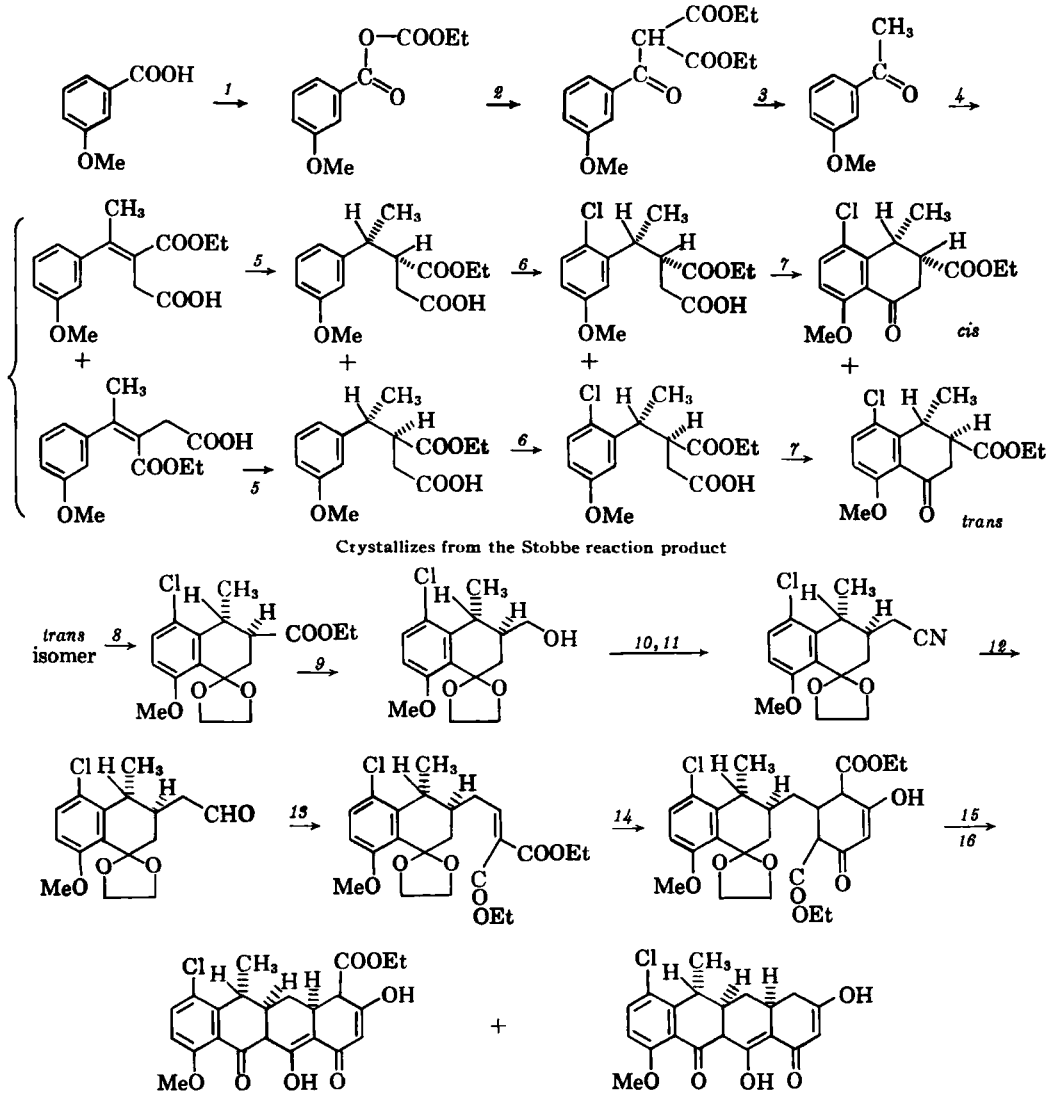
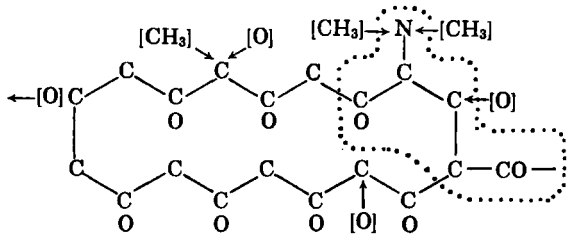


Fig. 1.—Synthesis of (±)-dedimethylamino-6,12a-dideoxy-decarboxamido-7-chlorotetracycline (101, 102, 162). Reagents: (*1*), ClCOOEt/N-methylmorpholine/benzene; (*2*), (EtOOC—$\bar{C}$H—COOEt) Mg^{++} ($\bar{O}$Et); (*3*), aq. H_2SO_4/AcOH/heat; (*4*), diethyl succinate/NaH; (*5*), H_2/Ni; (*6*), 1 equiv. Cl_2/CCl_4; (*7*), polyphosphoric acid; (*8*), ethylene glycol; (*9*), $LiAlH_4$; (*10*), methanesulfonyl chloride/pyridine; (*11*), KCN/dimethylformamide/water; (*12*), $LiAl(OEt)_3H$; (*13*), diethyl malonate/AcOH/piperidine; (*14*), Na^+(CH_3CO—CH—COOEt)/ether/reflux; (*15*), aq. HCl; (*16*), NaH/anisole.



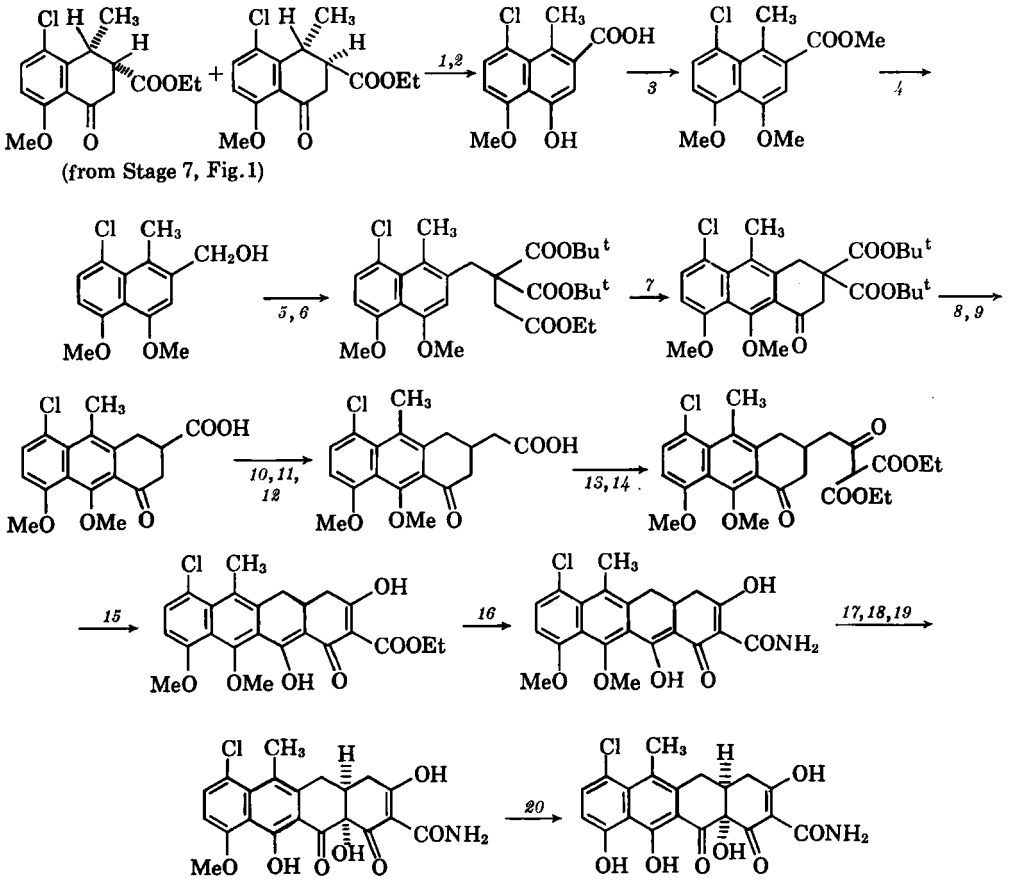
V

tetracycline contains 40% of the total radioactivity when glycine-2-^{14}C is used as substrate for *S. aureofaciens*, but carboxyl-^{14}C-glycine is not significantly utilized (88).

The use of mutant strains (59) to oxygenate anhydrotetracyclines has been mentioned, and current investigation of precursor activity possessed by other tetracycline degradation products might be extended to make available new biosynthetic tetracyclines from modified precursors, and from new mutant *Streptomycetes*.

TOTALLY SYNTHETIC TETRACYCLINE ANALOGS

Confirmatory syntheses of degradation products obtained during the structural elucidation



(from Stage 7, Fig. 1)

(and 12a-epimer)

Fig. 2.—Synthesis of (±)-dedimethylamino-12a-deoxy-5a,6-anhydro-7-chlorotetracycline (101) and its conversion into (±)-dedimethylamino-5a,6-anhydro-7-chlorotetracycline (103, 162). Reagents: (*1*), Br_2/ether/500-watt lamp; (*2*), NaOH/MeOH; (*3*), CH_2N_2; (*4*), $LiAlH_4$; (*5*), PBr_3; (6), Na^+(EtOOC—CH_2—$\bar{C}$(COOBu^t)$_2$); (*7*), polyphosphoric acid; (*8*), dil. aq. NaOH; (*9*), diethyl phthalate/170°; (*10*), PCl_5 or oxalyl chloride; (*11*), CH_2N_2; (*12*), benzyl alcohol/180°; (*13*), PCl_5; (*14*) Mg^{++}(EtOOC—$\bar{C}$H—COOEt)$_2$; (*15*), NaH/anisole; (*16*) NH_3/NaOMe—MeOH; (*17*), HCl/AcOH; (*18*), CH_2N_2; (*19*), $PhCO_3H/CHCl_3$; (*20*), HCl/AcOH.

of oxytetracycline and chlortetracycline were announced between 1951 and 1959 (89–96, 148, 152), and papers describing syntheses of simple model compounds date from 1956. Initial stages in projected total syntheses of tetracyclines were announced in 1957 by several research groups, and their continued efforts during later years were augmented by work in other laboratories; the objective has recently been realized by the total synthesis of (±)-6-demethyl-6-deoxytetracycline (97).

The complex stereochemistry and substitution of the tetracycline molecule has inspired many different conceptions of its total synthesis, each route representing a particular approach to the solution of problems of stereochemistry and synthesis. The most successful approach follows, in broad outline, the probable biogenetic route; various forms of the Claisen condensation are employed in the stepwise fusion of rings C, B, and A to a benzene derivative carrying the ring D substituents and a basis for the construction of ring C. The approach is exemplified by each of the syntheses outlined in Figs. 1–6; the products obtained by the routes in Figs. 2, 3, 4, and 5 were proved to be identical to the appropriate tetracycline degradation products. Although an adaptation of Woodward's synthesis (Fig. 5) has not yet been announced which leads to a naturally occurring 6-substituted tetracycline, and Muxfeldt's and Boothe's earlier routes (Figs. 2 and 3, respectively) yield dedimethylamino-anhydrotetracyclines, the major problems in this approach to the total synthesis have been overcome. Other research groups have reported initial stages in related syntheses, *e.g.*, synthesis of VI (92) (see also (95)), VII (98), and stereoisomers of VIII (99) (see also (161)), and model reactions have supplied useful guidance to crucial steps—the introduction of the N,N-dimethylglycine residue in Woodward's synthesis was earlier worked out, in principle, by Shemyakin's

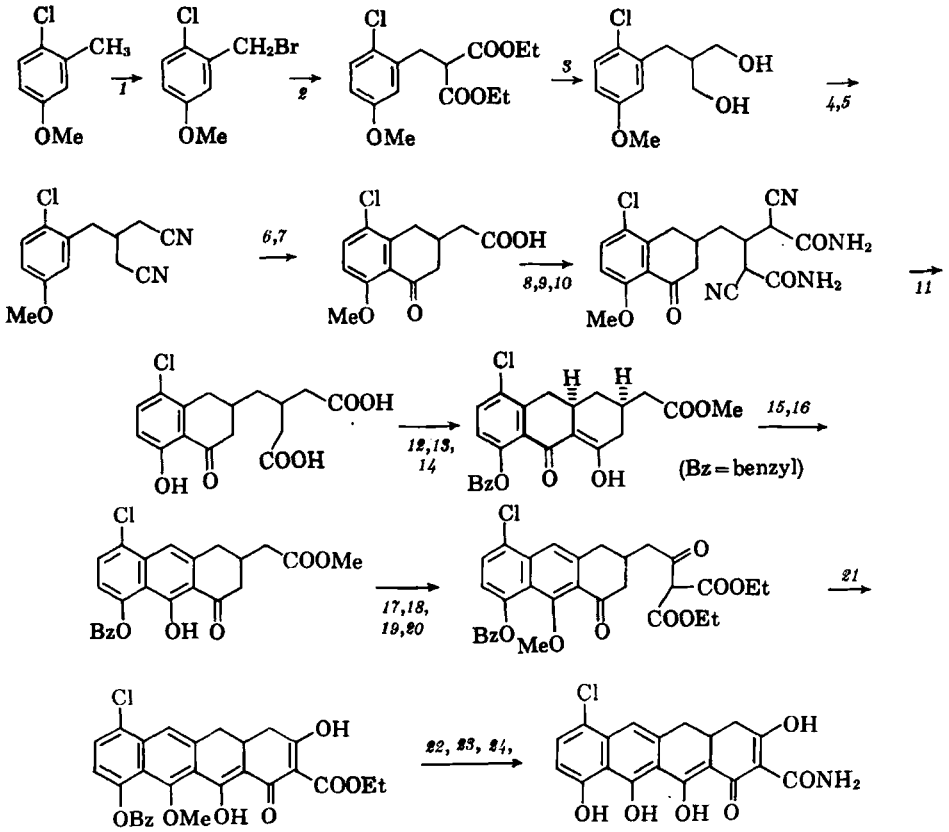
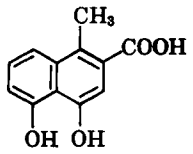
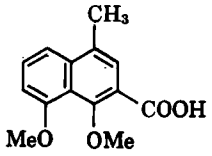


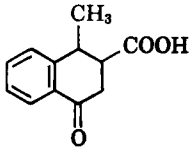
Fig. 3.—Synthesis of (±)-dedimethylamino-6-demethyl-12a-deoxy-5a,6-anhydro-tetracycline (104, 105, 135, 151). Reagents: (*1*), N-bromosuccinimide/peroxide; (*2*), Na^+(EtOOC—$\bar{C}$H—COOEt); (*3*), $LiAlH_4$; (*4*), methane sulfonyl chloride; (*5*), CN^-; (*6*), OH^-; (*7*), polyphosphoric acid; (*8*), oxalyl chloride; (*9*), Rosenmund reduction (5% Pd–$BaSO_4$); (*10*), cyanoacetamide/piperidine; (*11*), concd. HCl/AcOH; (*12*), benzyl chloride/boiling alkali; (*13*), $MeOH/H_2SO_4$; (*14*), NaH/toluene; (*15*), Br_2/NaOAc; (*16*), collidine (dehydrobromination); (*17*), Me_2SO_4/K_2CO_3; (*18*), mild alkaline hydrolysis; (*19*), ethyl chloroformate/NEt_3; (*20*), Mg^{++} ($\bar{O}$Et) (EtOOC—$\bar{C}$H—COOEt); (*21*), NaH/toluene; (*22*), H_2/10% Pd–C; (*23*), $HCOO^-NH_4^+$/140°; (*24*), boiling concd. HCl/AcOH.



VI



VII

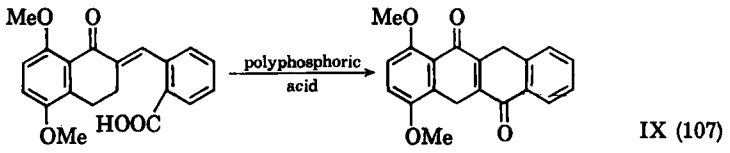


VIII

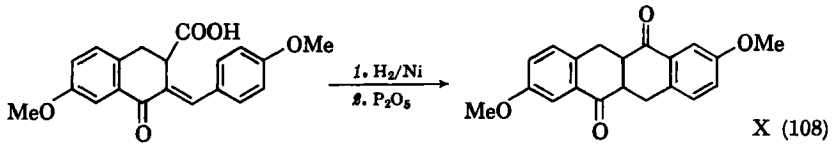
group (100), starting from cyclohexanone as a model.

Modification of each of the syntheses outlined above is easily visualized to yield many differently substituted tetracyclines, possibly with improved medical properties; and the outcome of other approaches to the total synthesis may provide alternative routes to modified tetracyclines.

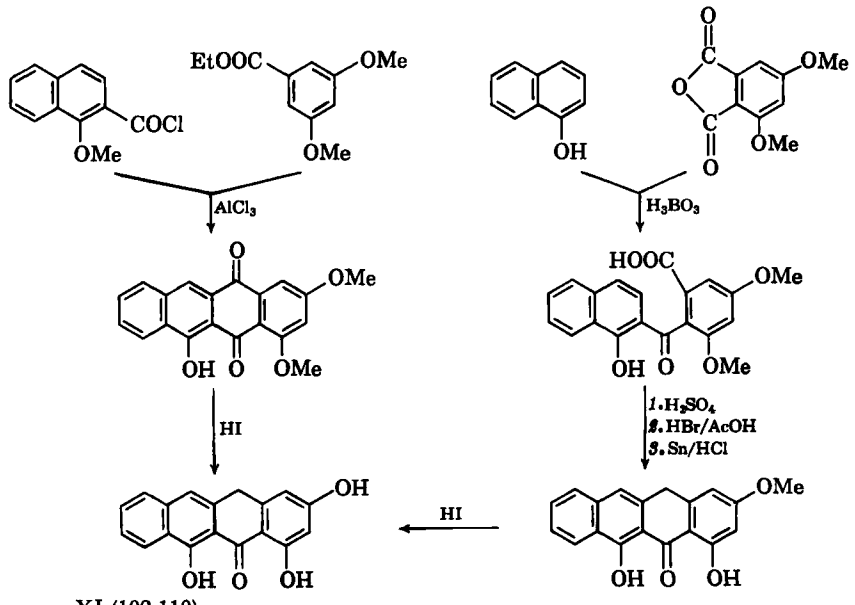
Naphthacene derivatives IX, X, and XI have been synthesized as more or less distant relatives of the antibiotics; compounds XI and XII of this group are closely similar to dedimethylamino-terrarubein, a degradation product of oxytetracycline (15). The naphthacene derivative XIII was found to possess less than 0.1% of the antimicrobial activity of chlortetracycline



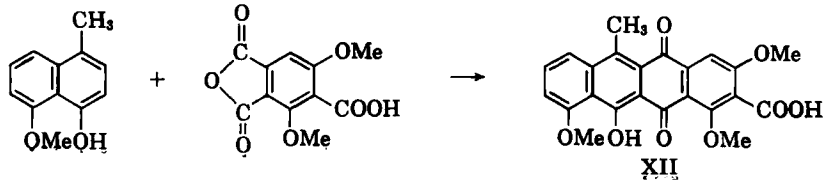
IX (107)



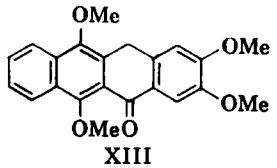
X (108)



XI (109,110)



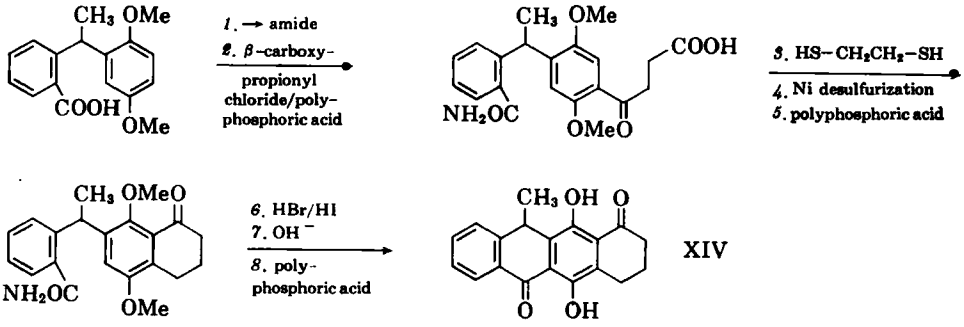
XII



XIII

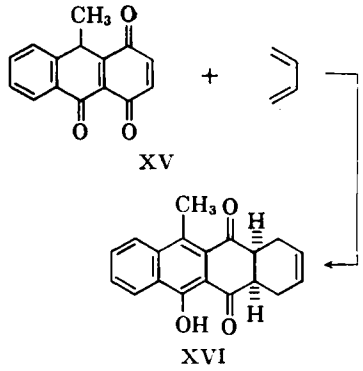
(111, 161); it is the only compound of this class whose antibiotic activity has been reported. The dark orange tetracycline analog XIV was built up as shown (112).

The more successful analogs are likely to be those which incorporate features of the stereo-



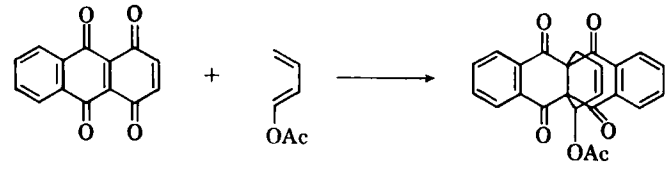
XIV

chemistry of the natural tetracyclines, and the Diels-Alder reaction has been used for the synthesis of a variety of compounds containing *cis*-fused rings corresponding to rings A and B. The tricyclic quinone XV has been reported (109, 113) to yield with butadiene the expected adduct XVI, although the same quinone was earlier (112) found to be unstable to Diels-Alder reaction conditions with butadiene.

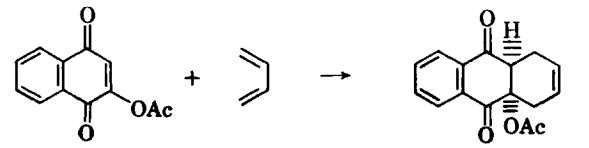


XV XVI

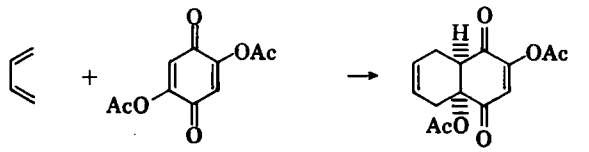
Quinazarinquinone XVII failed to yield the expected tetracycline analog with 1-acetoxybutadiene, but *cis-trans*-1,4-diacetoxybutadiene added



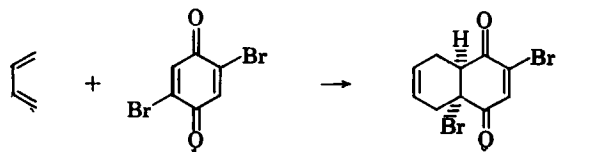
XVII



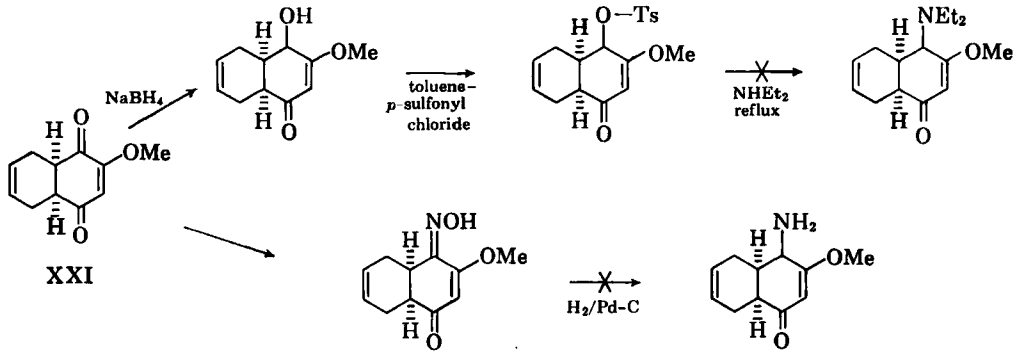
XVIII (115)



XIX (115)



XX (96)



normally (114). Related two- and three-ring model compounds have been prepared, some (XVIII, XIX) carrying an oxygen function at the ring junction. The unstable adduct XX could not be converted into XIX by treatment with silver acetate (96). One carbonyl group in the adduct XXI from butadiene and 2-methoxybenzoquinone can be selectively reduced, and the adduct can be converted into its mono-oxime; but efforts to introduce a nitrogen function corresponding to the ring A dimethylamino-substituent were not successful (116).

Nakamichi (117) has prepared adducts from *p*-toluquinol and a series of symmetrical and unsymmetrical dienes, and has reported that the adducts with isoprene and with cyclohexadiene showed marked *in vitro* activity.

The model compound XXII carries five oxygen functions, disposed in almost the same spatial arrangement as those of rings A and B of oxytetracycline, and may possess useful activity, in confirmation of the role played by the A- and B-ring substituents in conferring activity on the tetracycline molecule, when converted into its 2-carboxamide (118).

The Diels-Alder reaction has been applied also to the incorporation of the B-C ring-junction into intermediates potentially suitable for total synthesis of tetracyclines. Derivatives of juglone XXIII yield adducts XXIV with 1-acetoxybutadiene (119), with *cis-trans*-1,4-diacetoxybutadiene (120), and with 2- and 3-substituted butadienes (121), and one of the carbonyl groups in some of the various products is selectively

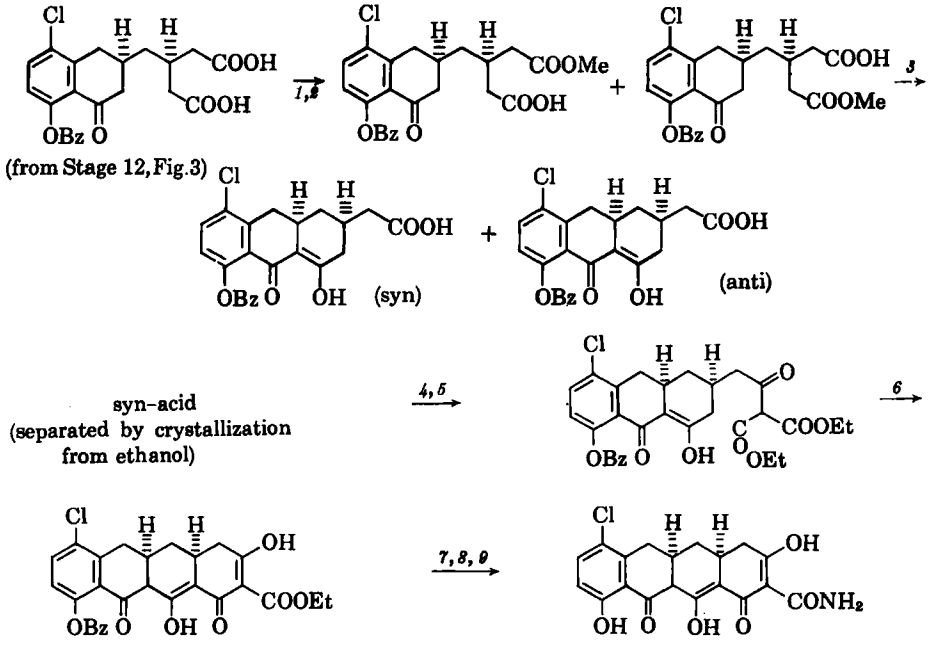
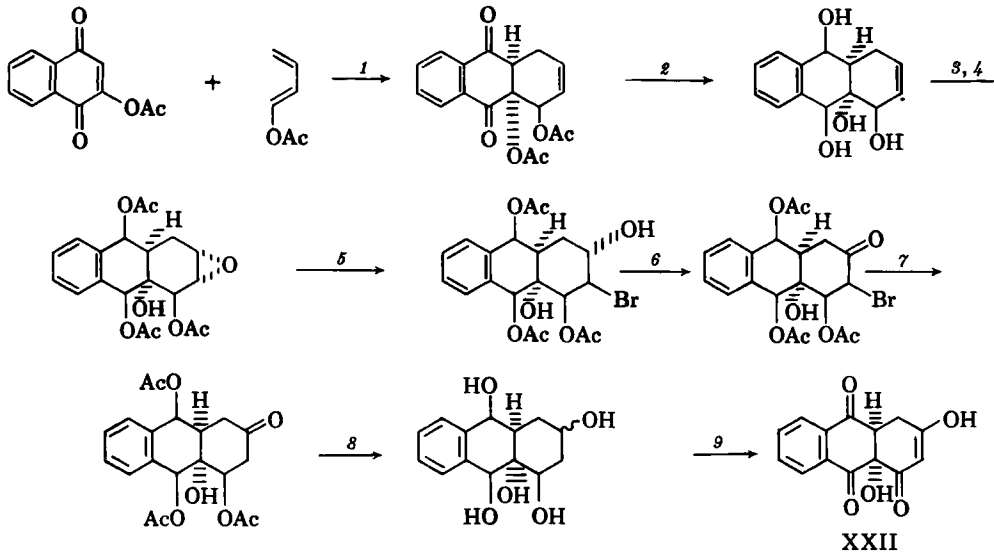
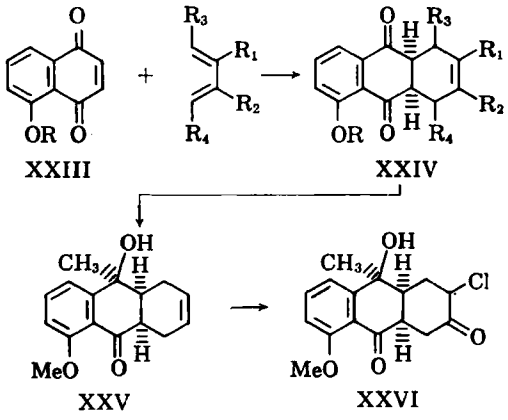


Fig. 4.—Synthesis of (±)-dedimethylamino-6-demethyl-6,12a-dideoxy-7-chlorotetracycline (106, 135). Reagents: (*1*), Ac_2O/reflux/1 hr.; (*2*), NaOMe/MeOH; (*3*), NaH/toluene; (*4*), ClCOOEt/NEt_3; (*5*) Mg^{++} ($EtOOC—\overset{|}{C}H—\overset{|}{C}OOEt_2$); (*6*), NaH/toluene; (*7*), H_2/10% Pd-C; (*8*), $HCOO^-NH_4^+$/140°; (*9*), Aq.HCl.



XXII

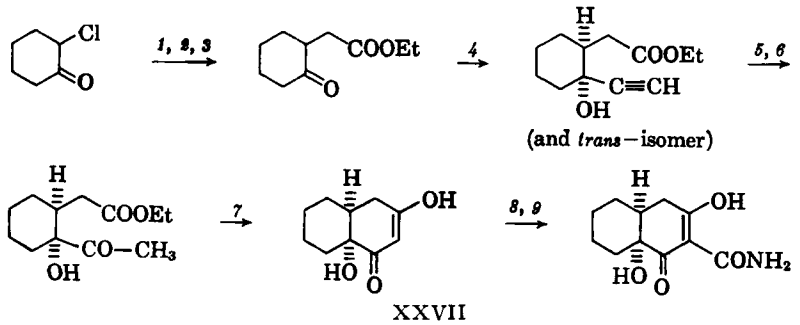
Reagents: (*1*), benzene, reflux; (*2*), $LiAlH_4$; (*3*), Ac_2O/pyridine; (*4*), monoperphthalic acid/dioxane; (*5*), 48% aq. HBr/$CHCl_3$; (*6*), CrO_3/AcOH; (*7*), Zn/AcOH; (*8*), $LiAlH_4$; (*9*), CrO_3, acetone.



XXIII XXIV XXV XXVI

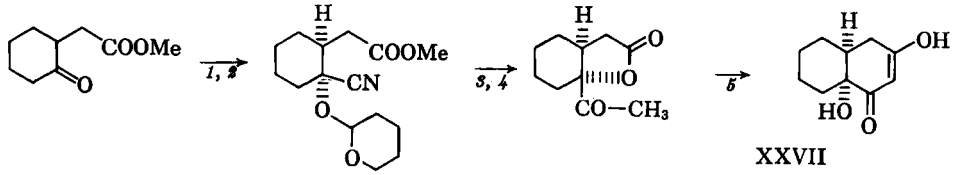
attacked during their reaction with one equivalent of methyl magnesium iodide (121, 122), yielding the useful intermediate XXV from the adduct XXIV (R = OMe, R_1, R_2, R_3, R_4 = H). Further elaboration (123) of XXV has yielded the chloroketone XXVI (for the synthesis of a tetracycline from an analog of XXV see Fig. 6), which appears suitable for use in the route developed for the synthesis, from 2-chlorocyclohexanone, of a model compound carrying the ring A substituents with the exception of the dimethylamino group (124, 125, 164).

The overall route should thus yield (±)-dedimethylamino-12-deoxy-tetracyclines with the natural stereochemistry. New routes to XXVII have been reported recently (126) by the Moscow workers, who have also achieved the synthesis



XXVII

Reagents: (*1*), $Na^+(EtOOC—\bar{C}H—COOEt)$; (*2*), hydrolysis; (*3*) decarboxylation; (*4*), $Na^+\ \bar{C}{\equiv}CH$; (*5*), $Hg(OAc)_2$; (*6*), H_2S; (*7*), NaOEt/EtOH/room temp.; (*8*), AcNCO; (*9*), NH_3/MeOH.



XXVII

Reagents: (*1*), $Me_2C(OH)CN/MeOH/K_2CO_3/20°$; (*2*), 2,3-dihydropyran/$POCl_3$; (*3*), MeMgI; (*4*), aq. HCl; (*5*), NaOEt.

of its 4-dimethylamino-analog XXVIII. The synthesis of a tetracycline, outlined in Fig 6, also involves the cyanohydrin-lactone sequence exemplified in the syntheses of the model compounds XXVII and XXVIII.

The possibility that the naphthoquinone-butadiene adducts XXIV might be selectively reduced, to yield tricyclic intermediates suitable for the synthesis of 6-demethyltetracyclines, has also been investigated (126). 1,4,4a,9a-Tetrahydroanthraquinone (XXIX) yields the ketol XXX with one equivalent of $LiAlH_4$, and further elaboration has yielded the epoxide XXXI suitable for the route outlined in Fig. 6.

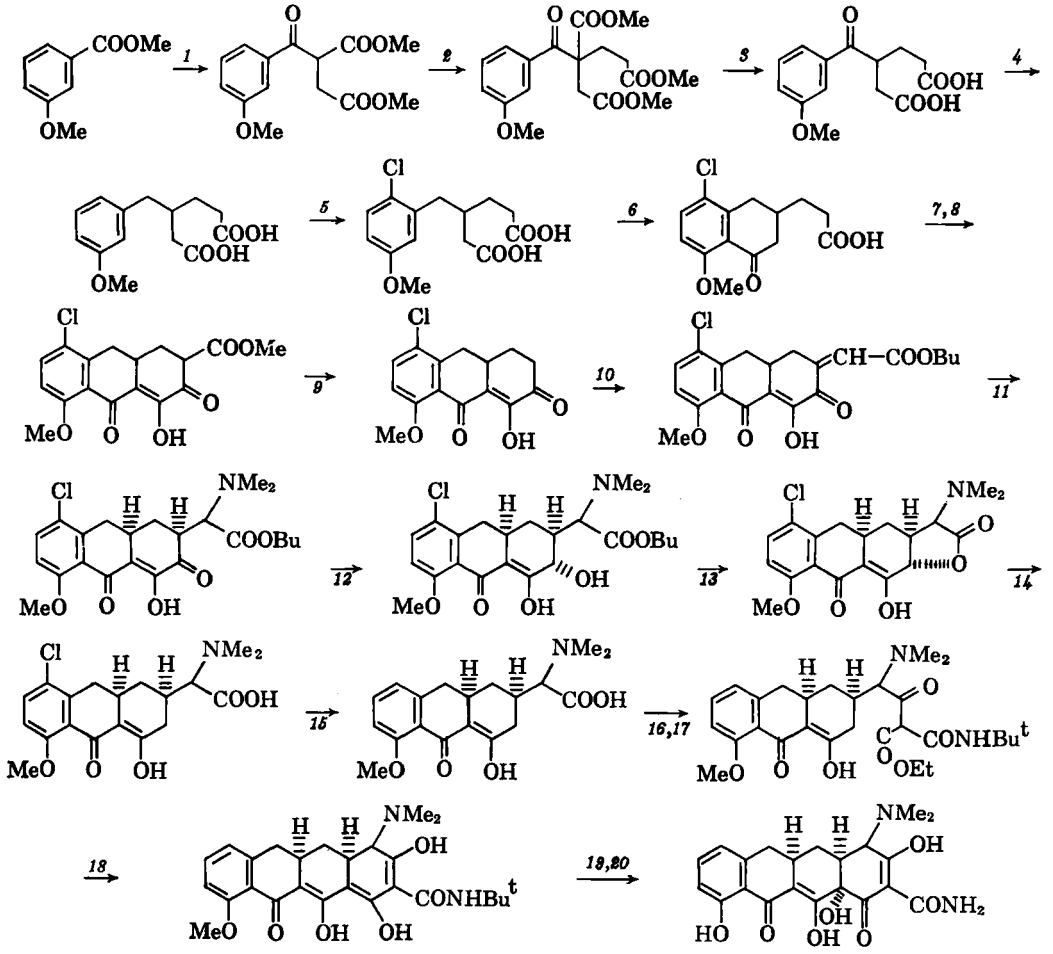
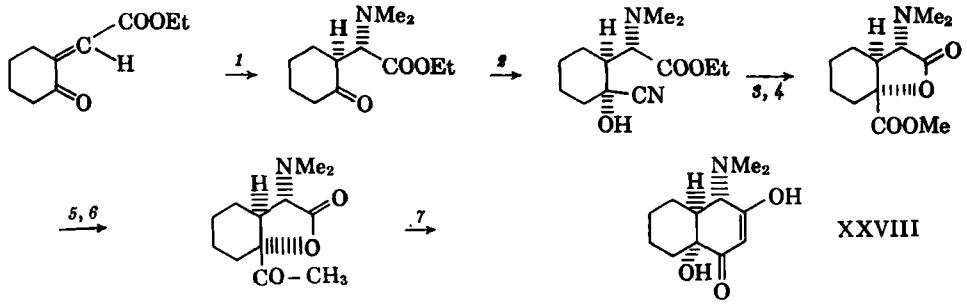
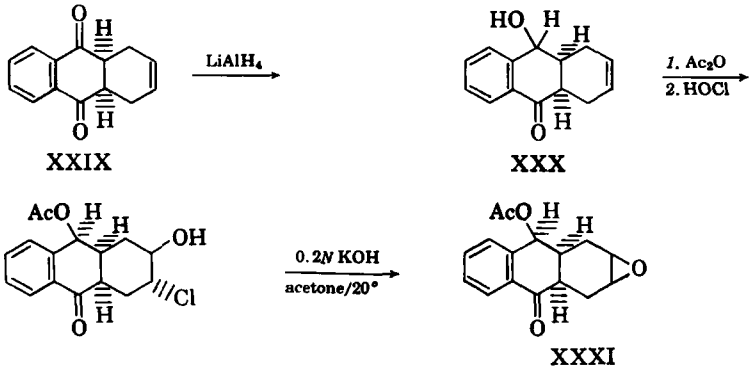


Fig. 5.—Synthesis of (±)-6-demethyl-6-deoxytetracycline (97). Reagents: (*1*), dimethyl succinate/NaH/dimethylformamide; (*2*), methyl acrylate/Triton B; (*3*), hot aq. H_2SO_4/AcOH; (*4*), H_2/Pd–C/AcOH/200 p.s.i.; (*5*), Cl_2/AcOH/15°; (*6*), HF/15°; (*7*), esterify; (*8*), dimethyl oxalate/1 equiv. MeOH/NaH/dimethylformamide; (*9*), hot aq. HCl/AcOH; (*10*), Mg^{++} $(\bar{O}Me)_2$/*n*-butyl glyoxalate/toluene; (*11*), Me_2NH/−10°; (*12*), $NaBH_4$/diglyme/low temp.; (*13*), toluene sulfonic acid/toluene; (*14*), Zn/HCOOH; (*15*), H_2/Pd–C/EtOH/NEt_3; (*16*), $ClCOOPr^i$; (*17*), Mg^{++} $(EtOOC—\bar{C}H—CONHBu^t)_2$; (*18*), NaH/dimethylformamide/120°; (*19*), hot 48% aq. HBr; (*20*), $CeCl_2/O_2$/dimethylformamide/MeOH/pH 5.



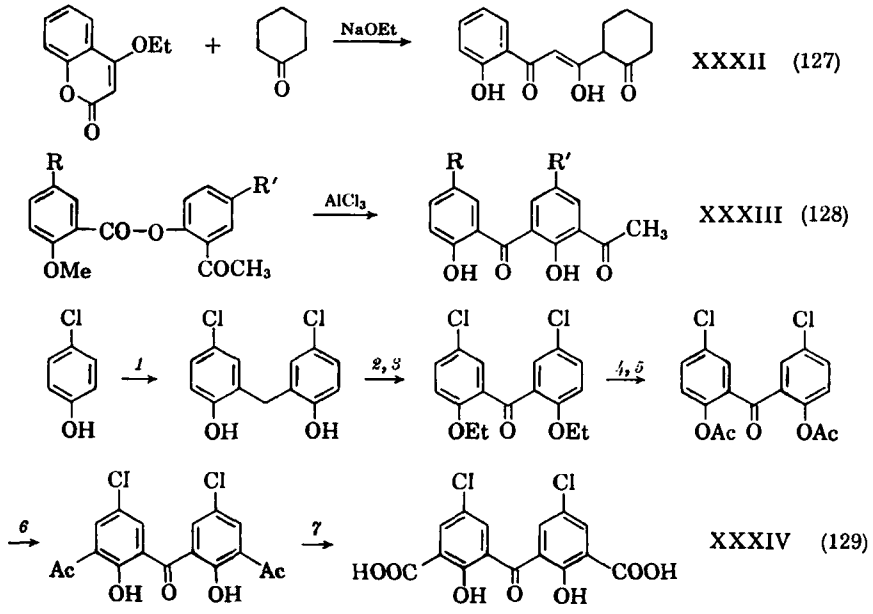
XXVIII

Reagents: (*1*), Me_2NH/hexane/0°; (*2*), $Me_2C(OH)CN/MeOH/K_2CO_3$/20°; (*3*), concd. HCl; (*4*), esterify; (*5*), Bu^tOAc/Bu^tOK; (*6*), aq. HCl; (*7*), NaOEt/EtOH/20°.



XXIX XXX XXXI

Several model compounds, XXXII–XXXIV, containing the chelating carbonyl and enolic oxygen functions of the tetracycline molecule have been synthesized; no activities have been reported. The tetrahydroanthracene XXXV possesses appreciable activity (30 oxytetracycline units per milligram) (130, 131, 132); its mono-, di-, and tri-chloro- and bromo-derivatives



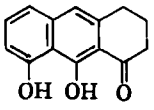
XXXII (127)

XXXIII (128)

XXXIV (129)

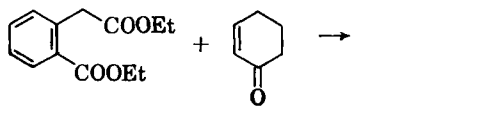
Reagents: (*1*), $CH_2O/MeOH/H_2SO_4$; (*2*), Et_2SO_4; (*3*), CrO_3/AcOH; (*4*), $AlCl_3/CS_2$; (*5*), Ac_2O/H_2SO_4 (*6*), $AlCl_3$/170°; (*7*), NaOI.

(substituents introduced into the 5, 7, and 10 positions by direct halogenation) are more stable to oxidation, and also possess antimicrobial activity (133).

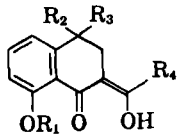


XXXV

Other related di- and tri-cyclic analogs of this part of the molecule have been prepared, in some cases particularly for pKa studies, and similarly-constructed intermediates in the synthetic routes outlined in Figs. 1–6 may possess activity comparable with that of XXXV. Trioxo-octahydroanthracenes and related naphthacenes (Fig. 4) are claimed to be generally useful as chelating, complexing, and sequestering agents for poly-



XXXVI (138)



XXXVII

(136,15) $R_1 = R_2 = R_3 = H$; $R_4 = CH_3$
(8) R_1 = Et; $R_2 = R_3 = H$; R_4 = cyclohexyl
(8) R_1 = benzyl; $R_2 = R_3 = H$; R_4 = Me
(137) $R_1 = R_2$ = Me; R_3 = OH; R_4 = Et

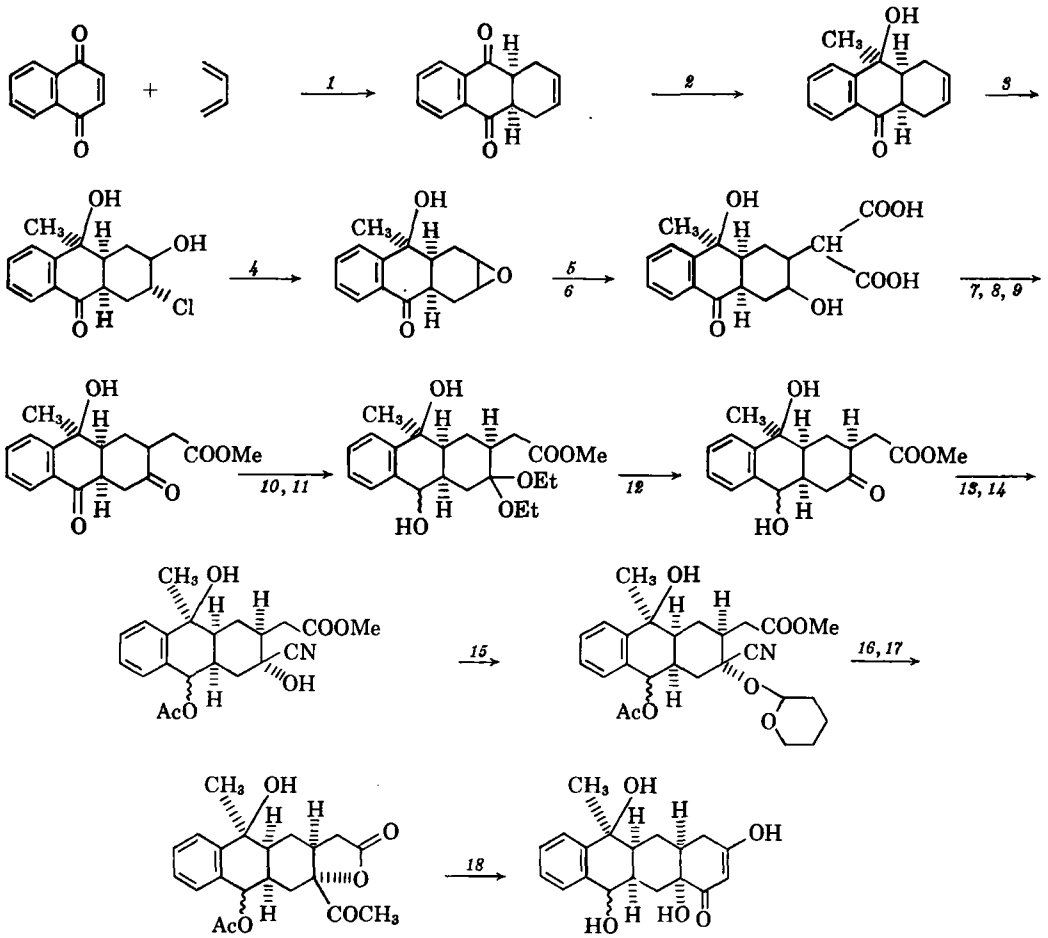
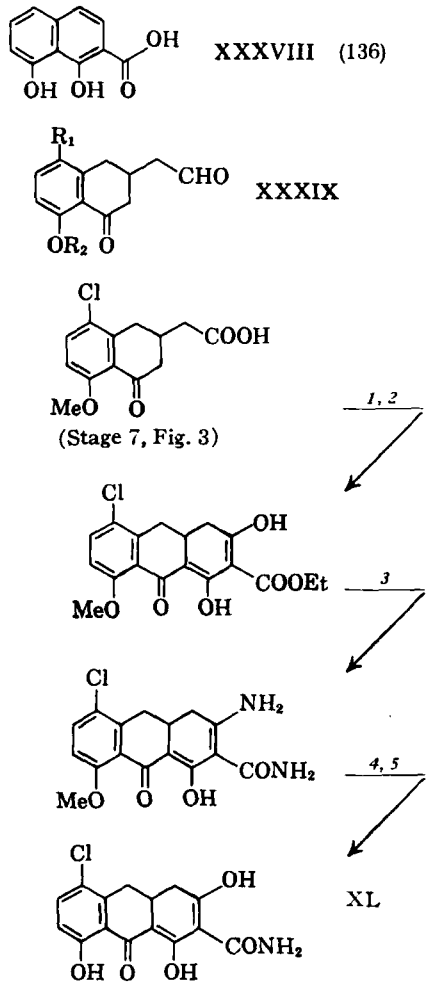
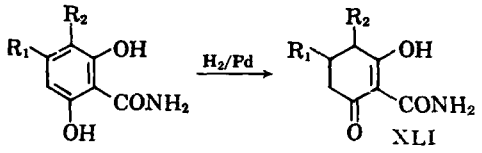


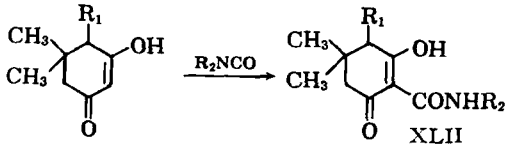
Fig. 6.—Synthesis of (±)-dedimethylamino-decarboxamido-10,12-dideoxytetracycline (121, 123, 126). Reagents: (*1*), 100°; (*2*), 1 equiv. MeMgI; (*3*), Bu^{t}OCl; (*4*), aq. KOH/dioxan; (*5*), Na^+ (EtOOC—CH—COOEt); (*6*), OH^-; (*7*), pyridine and piperidine/120°; (*8*), CrO_3/AcOH/35°/1 hr.; (*9*), CH_2N_2; (*10*), $HC(OEt)_3$; (*11*), $NaBH_4$; (*12*), hydrolysis; (*13*), Ac_2O; (*14*), acetone cyanhydrin/catalytic quantity of NH_3; (*15*), dihydropyran/$POCl_3$; (*16*), MeMgI; (*17*), warm AcOH; (*18*), NaOEt/EtOH.



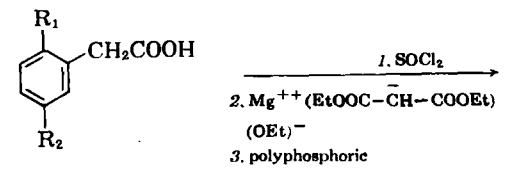
Reagents: (*1*), oxalyl chloride; (*2*), $Na^+(EtOOC—CH—COOEt)$; (*3*), NH_3/MeOH/80°/pressure/5 hr.; (*4*), 4 *N* aq. HCl; (*5*), HBr—AcOH.



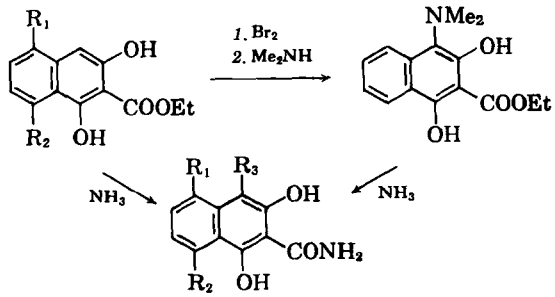
$R_1 = R_2 = H$	(8)
$R_1 = H$; $R_2 = NH_2$	(140)
$R_1 = H$; $R_2 = NMe_2$	(141)
$R_1 = Me$; $R_2 = H$	(138)



(a) $R_1 = R_2 = H$	(125)
(b) $R_1 = H$; $R_2 = Ph$	(125)
(c) $R_1 = Br$; $R_2 = Ph$	(140, 141)
(d) $R_1 = H$; $R_2 = Ac$	(125)
(e) R_1 = piperidino-; $R_2 = Ph$	(140, 141)



$R_1 = R_2 = H$
$R_1 = OMe$; $R_2 = H$
$R_1 = R_2 = OMe$



$R_1 = R_2 = R_3 = H$
$R_1 = OMe$; $R_2 = R_3 = H$
$R_1 = R_2 = OMe$; $R_3 = H$
$R_1 = R_2 = H$; $R_3 = NMe_2$
(8, 142, 144)

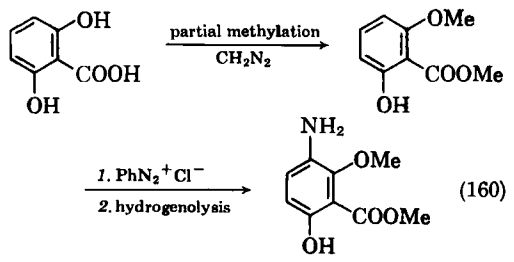
valent metal ions (134); dioxo-octahydroanthracene acetic acid derivatives are similarly effective (135), and tetraloneacetaldehyde derivatives (*e.g.*, XXXIX) possess fungicidal properties (149).

The model compound XL incorporates the main features of rings A, C, and D of 7-chloro-6-demethyl-6-deoxy-tetracycline, and of rings A, B, and D of chlortetracycline, but it is quite inactive (139). It is structurally related to the active tetrahydroanthracene XXXV, and the reason for the differing activities of these two compounds possibly lies in the differing potential chelation sites presented by them to metal ions.

Several ring A analogs are available, compounds XLII (b, c, and e) possessing weak tetracycline activity.

Appropriately substituted benzene and naphthalene models for ring A have been synthesized.

The minimum requirements for activity (discussed in the section of this review devoted to *The Mode of Action of the Tetracyclines*) revealed by antibiotic assays of tetracyclines derived from the natural products are a useful guide to future syntheses of simpler analogs. However, few of the available totally synthetic tetracycline analogs appear to have been tested; those bearing structural similarities with the



(160)

A-ring analogs, XLII, the BCD-rings analog, XXXV, and the *p*-toluquinol-isoprene and *p*-toluquinol-cyclohexadiene adducts (117) may possess similar activity.

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Esters of Bicyclic Aminoalcohols as Potential Anticholinergics III

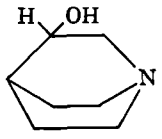
Synthesis of Some Isomeric Hydroxy-1-azabicyclononanes and Certain of Their Esters†

By MICHAEL J. MARTELL, JR.‡ and TAITO O. SOINE

Attempts to improve upon the literature preparation of 3-oxo-1-azabicyclo[3.2.2]-nonane failed. The previously unknown aminoalcohol 3-hydroxy-1-azabicyclo-[3.3.1]nonane (VI) was synthesized in good yield. The described synthesis of 8-hydroxy-1-azabicyclo[4.3.0]nonane (VII) was improved considerably and a series of nineteen esters of VI and VII was prepared using five acids. The pharmacological testing of the esters is reported.

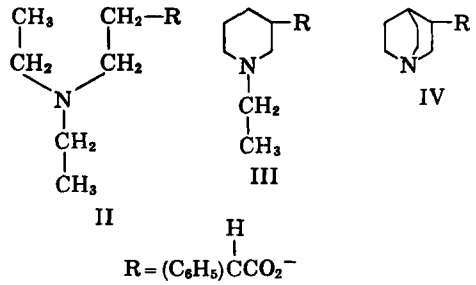
THESE laboratories have been engaged for some time (1) in the synthesis of esters of bicyclic aminoalcohols as potential spasmolytics. The paucity of information in this area has been discussed in the earlier papers.

Referring to the work of Sternbach and Kaiser (2), one can see from models that 3-quinuclidinol is arranged in a compact, rigid cage-type structure, the piperidine ring of which exists in the boat form (I).



I

The inflexibility of the molecule may have some bearing on the marked spasmolytic activity of its esters when one considers the differences in activity between 2-diethylaminoethyl diphenylacetate[1] (II), piperidolate[2] (III), and the diphenylacetate of 3-quinuclidinol (IV). 2-Diethylaminoethyl diphenylacetate has roughly the same order of activity as piperidolate (3), and is about 1/4 as active as atropine (4). However, racemic IV is equal in activity to atropine and the (−) enantiomorph is twice as active.



II III IV

R = $(C_6H_5)CCO_2^-$

To further explore the subtle effects of molecular flexibility on spasmolytic activity, it was decided to synthesize the aminoalcohols V, VI, and VII, if possible, and to prepare a representative selection of esters from them to be tested for anticholinergic activity. These isomeric bicyclic aminoalcohols are all β-aminoalcohols, esters of which have been shown to be the most active, particularly in the bicyclic series (5).

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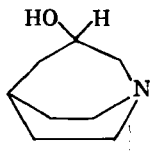
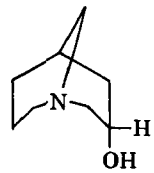
[1] Marketed as Trasentine by Ciba Pharmaceutica Products Inc., Summit, N. J.

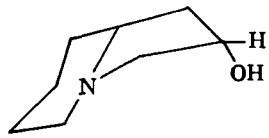
[2] Marketed as Dactil by Lakeside Laboratories, Inc., Milwaukee, Wis.



V



VI



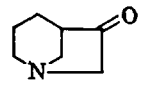
VII

The stereochemistry of 3-hydroxy-1-azabicyclo-[3.2.2]nonane (V) [and quinuclidine (6)], as well as 3-hydroxy-1-azabicyclo[3.3.1]nonane[3] (7, 8) (VI), and 8-hydroxy-1-azabicyclo[4.3.0]nonane[4] (VII) (as a special azahydrindane (9)) has been adequately discussed in the literature. Suffice it to say that, as one passes from V to VII, the molecular flexibility is increased among these isomers.

The aminoalcohols in this study were prepared by the Dieckmann synthesis for aminoketones possessing a bridgehead nitrogen (10, 11). It was found impractical, according to this method, to prepare suitable quantities of V and esters of this aminoalcohol were not prepared. The other two aminoalcohols, VI and VII, were prepared in good yield, however.

For these aminoalcohols the three isomeric pyridylacetic esters were required as key intermediates in good quantity.[5]

Although VII had been prepared previously (12), the synthesis was impracticable in part. Ethyl 2-pyridylacetate was best prepared by the method of Winterfeld and Flick (13). Reduction of the pyridine ring was carried out as described (1), followed by alkylation with ethyl chloroacetate in the presence of anhydrous potassium carbonate (12). The diester thus obtained was best cyclized to the aminoketone using high dilution methods and employing potassium *tert*-butylate in a nitrogen atmosphere (14, 15). Reduction of the aminoketone to the alcohol in water by sodium borohydride was accomplished in nearly quantitative yield. Although the aminoketone was rapidly air-oxidized, the aminoalcohol was quite stable. The reduction product appears identical with the product prepared by Clemo and Metcalf (12) by sodium and alcohol reduction.[6]



i

Although several syntheses of 3-pyridylacetic ester are published in the literature, none appeared to be suitable without modification as an intermediate to VI. A sample of 3-pyridylthioacetmorpholide (16, 17) prepared from 3-acetylpyridine (18, 19) was converted directly to ethyl 3-pyridylacetate. Refluxing the thiomorpholide with concentrated hydrochloric acid (18) and replacement of the acid with alcohol and sulfuric acid gave good yields of the desired ester. Quaternization of the ester with ethylbromoacetate proceeded smoothly as did the subsequent reduction to the diester. Cyclization with potassium *tert*-butylate as described (14) gave the desired aminoketone in 74% yield. Sodium borohydride reduction in water afforded only one isomer.[7]

The preparation of the aminoketone corresponding to V according to the procedure of Leonard and co-workers (14) failed to give better yields than the 0.7% conversion reported in the Dieckmann reaction. Attempts to improve the yield by employing other catalysts or changing reaction conditions were fruitless. It would seem that perhaps the three carbon atom bridge increases greatly the extent of nonbonded interactions in the piperidine ring over that of quinuclidine (6).

Substantial improvements in the published methods for the preparation of ethyl 4-pyridylacetate were developed and 4-pyridylthioacetmorpholide was conveniently prepared by the reaction of 4-vinylpyridine, sulfur, and morpholine in the presence of hydroquinone. The thiomorpholide was converted directly to the ester by simply refluxing with equal weights of

[3] Although *Chemical Abstracts* catalogs this nucleus under the name 1-isogranatanine, in accordance with the nomenclature suggested by McElvain and Adams, *J. Am. Chem. Soc.*, **45**, 2744(1923), the authors prefer the 1-azabicyclo terminology to maintain consistency and to demonstrate the correlation among isomers.

[4] *Chemical Abstracts* prefers the name octahydropyrrocoline for this nucleus (see above). Other trivial names include piperolidine, indolizidine, and δ-coniceine.

[5] The 2, 3, and 4-pyridylacetic acids are now commercially available (Aldrich Chemical Co.). For a review of methods of synthesizing the isomeric pyridylacetic acids and their derivatives see M. J. Martell, Jr., Ph.D. thesis (University of Minnesota, 1958).

[6] These authors also reported the isolation of another "isomeric aminoalcohol" as a by-product of the Clemmensen reduction of the aminoketone. However, such a product seems unlikely, and, although no reinvestigation of this result was undertaken, it would seem that cleavage during reduction had taken place, probably to 2-piperidylacetone, isomeric with the aminoalcohol. Leonard and co-workers (14) have shown that *i* is cleaved during Clemmensen reduction to 3-acetylpiperidine.

[7] The reduction of bicyclic aminoketones of the bicyclo-[3.3.1]type has been investigated recently and excellently reviewed [Zirkle, C. L., Gerno, F. R., Pavloff, A. M., and Burger, A., *J. Org. Chem.*, **26**, 395(1961)] and shows that attack with large reducing groups is from the unhindered side giving the less thermodynamically stable axial conformer, which indeed VI may well be. Reduction with sodium and amyl alcohol gives the more stable equatorial conformer. We are presently investigating this reduction in greater detail.

absolute alcohol and sulfuric acid (23). Formation of the quaternary salt and subsequent reactions followed those described by Leonard, *et al.* (14).

The acid chloride method, as described by Counsell and Soine, was used for the preparation of the esters of diphenylacetic and xanthene-9-carboxylic acids. Transesterification in *n*-heptane, using a trace of sodium hydride as catalyst, conveniently transformed the methyl esters of benzilic, phenyl-2-thienylglycolic, and 9-hydroxyfluorene-9-carboxylic acids to the corresponding esters of the respective aminoalcohols.

Published methods (20, 21) for the synthesis of phenyl-2-thienylglycolic acid were unsatisfactory for our purposes and therefore a direct synthesis of its methyl ester was developed. Thus, the action of 2-thienyl magnesium bromide on methyl phenylglyoxylate gave fair yields of methyl phenyl-2-thienylglycolate.

Examination of the pharmacological data presented in Tables I and II readily reveals that whatever differences in activity exist between corresponding esters of VI and VII are small indeed, and lie well within the errors presented by the testing methods. The compounds, however, are quite potent anticholinergic agents.

All of the esters were tested in Shay (pyloric ligated) rats (22) and their effects on gastric secretion noted. Only five esters were either sufficiently active or well absorbed orally to demonstrate significant activity. The benzilate and xanthene-9-carboxylate methobromides of VII showed minimal action on gastric acid and no effect on gastric secretion volume at 40 mg./Kg. The phenyl-2-thienylglycolate methobromide of VII had minimal action on both acid and volume at 20 mg./Kg. The phenyl-2-thienylglycolates of VI showed unusually high activity. The methobromide was effective at only 40 mg./Kg. but the hydrochloride was active at 10 mg./Kg. These last two compounds were tested in Heidenhain denervated and Thomas innervated chronic fistula dogs, and the effect on the gastric secretion observed. The methobromide in oral doses of 0.1–0.25 mg./Kg. produced slightly delayed onset of acid secretion. However 0.5 mg./Kg. caused suppression of gastric acid output by at least 50% in two dogs. At 0.05–0.1 mg./Kg., taken intravenously, the acid secretion was completely suppressed in two dogs. The hydrochloride in dogs was very potent. Oral doses of 0.5 mg./Kg. completely inhibited secretion of acid for 5 hours in 4 dogs (3 denervated, 1 innervated), but serious side effects probably resulting from central stimulation (5) were observed. At doses of 0.1 mg./Kg., side effects were reduced and the gastric secretion was still abolished for at least 5 hours. In this type of test, atropine abolishes gastric secretion at about 0.065 mg./Kg.[8]

EXPERIMENTAL[9]

4-Pyridylthioacetmorpholide.—A mixture of redistilled morpholine (45.7 Gm., 0.525 mole), freshly distilled 4-vinylpyridine[10] (50.0 Gm., 0.475 mole) containing 1% of hydroquinone, and sulfur (31.0 Gm., 0.95 Gm. atom) were placed in a 200-ml. flask equipped with a reflux condenser. The sulfur dissolved and the temperature of the solution rose to 110°. The solution was then heated at 160° for 2 hours, during which time hydrogen sulfide evolved. The solution was poured into cracked ice, stirred until solid, filtered, pressed as dry as possible, washed with ice water, dried and recrystallized from absolute ethanol. This procedure gave 56.0 Gm. (53%) of slightly impure material, m.p. 103–106° [m.p. 104–105.5° lit. (17)].

Ethyl 3-Pyridylacetate.—3-Pyridylthioacetmorpholide (195 Gm., 0.88 mole) (16, 17) was dissolved in concentrated hydrochloric acid (200 ml.) and the solution refluxed for 2.5 hours. The solution was cooled, filtered until clear, and evaporated to dryness *in vacuo*. The residue was dissolved in absolute ethanol (400 ml.) and the solvent distilled under reduced pressure to remove traces of water. The solid was again dissolved in absolute ethanol (500 ml.) and concentrated sulfuric acid (200 ml.) was added with cooling. The solution was allowed to stand for 24 hours at room temperature, after which it was refluxed for 1 hour. The cooled solution was poured slowly with stirring into 600 ml. of ice-cold concentrated ammonium hydroxide solution in an ice bath and the ester extracted with five 150-ml. portions of ether. The ether extracts were dried over anhydrous sodium sulfate and the solvent removed *in vacuo*. The product was distilled to give 92.5 Gm. (64%) of a light greenish liquid, b.p. 65° (0.1 mm.), n_D^{20} 1.5000 [b.p. 121–122° (10 mm.), lit. (17)].

Diethyl Pyridinium-1,3-diacetate Bromide.—Ethyl 3-pyridyl acetate (92.5 Gm., 0.56 mole), ethyl bromoacetate (94.0 Gm., 0.56 mole), and absolute ether (200 ml.) containing absolute ethanol (10 ml.) were mixed, briefly stirred, and left at room temperature for 24 hours. The oil that separated solidified upon scratching. The solid was separated by filtration, washed well with anhydrous ether, and dried to give 176 Gm. (94.5%) of product suitable for reduction. A sample was crystallized

[8] Dr. T. M. Lin, Eli Lilly and Co., personal communication.

[9] All melting points were determined on a calibrated Kofler micro hot stage apparatus unless otherwise specified, in which case they are uncorrected as are the boiling points. The microanalyses were performed by Wm. Kuryla and Mrs. Olga Hamerston of the Microanalytical Laboratories, School of Chemistry, University of Minnesota, or by Drs. Weiler and Strauss, Microanalytical Laboratories, Oxford, England. The infrared spectra were determined by the Spectroscopy Laboratory, School of Chemistry, University of Minnesota, on a Perkin-Elmer model 21 doublebeam spectrophotometer using sodium chloride optics.

[10] From the Reilly Tar and Chemical Co., Indianapolis, Ind.

several times from butanone to give hygroscopic colorless prisms, m.p. 81–82°.

Anal.—Calcd. for $C_{13}H_{18}BrNO_4$: C, 47.00; H, 5.46. Found: C, 47.27; H, 5.49.

Diethyl Piperidyl-1,3-diacetate.—Crude diethyl pyridinium-1,3-diacetate bromide (50 Gm., 0.15 mole) in glacial acetic acid (150 ml.) and water (50 ml.) was hydrogenated at 2–3 Atm. at room temperature, using 0.7 Gm. of platinum oxide catalyst. After uptake of hydrogen had ceased, the catalyst was removed by filtration and the solution evaporated to a syrup *in vacuo*. The syrup was neutralized with 25% aqueous sodium hydroxide solution and the diester extracted with five 50-ml. portions of ether. The ether extracts were dried over anhydrous sodium sulfate and the ether removed under reduced pressure. The residue was distilled to give 33.6 Gm. (87% yield) of product as a colorless oil, b.p. 110–111° (0.15 mm.), n_D^{25} 1.4607.

Anal.—Calcd. for $C_{13}H_{23}NO_4$: C, 60.67; H, 9.01. Found: C, 60.65; H, 8.97.

The picrate was formed in and recrystallized from absolute ethanol, m.p. 92–93°.

Anal.—Calcd. for $C_{19}H_{26}N_4O_{11}$: C, 46.92; H, 5.39. Found: C, 46.90; H, 5.53.

3 - Oxo - 1 - azabicyclo[3.3.1]nonane. — Anhydrous xylene (2500 ml.) and freshly trimmed potassium (45.6 Gm., 1.17 Gm. atoms) were placed in a 5-L. three-neck flask, equipped with a mechanical stirrer, reflux condenser fitted with a drying tube, and a dropping funnel. The xylene was heated and the potassium pulverized with vigorous stirring under a stream of dry nitrogen, which was maintained throughout the reaction. The heat was removed and *tert*-butyl alcohol (freshly distilled from calcium hydride) (325 ml., 3.5 moles) was added at such a rate as to maintain a gentle reflux. The mixture was then stirred and refluxed until all the potassium had reacted. The excess alcohol was removed by collecting 1200 ml. of azeotrope by means of a Dean-Stark trap. A solution of diethyl piperidyl-1,3-diacetate (120 Gm., 0.47 mole) in anhydrous xylene (800 ml.) was added over a period of 10 hours with vigorous stirring followed by a period of 20 hours of refluxing and stirring, the ethanol-xylene azeotrope being removed the entire time. The mixture, which by now was reduced in volume to about 1 L. was cooled in ice, and 400 ml. of 6 *N* hydrochloric acid was added with vigorous stirring. The xylene layer was extracted twice more with 200-ml. portions of 6 *N* acid, and the combined acid extracts refluxed for 8 hours. The cooled solution was basified to pH 9 with 50% aqueous potassium hydroxide, with cooling, saturated with solid potassium carbonate, and extracted with six 200-ml. portions of benzene. The combined benzene extracts were dried and the solvent removed at slightly reduced pressure. The solid residue was sublimed at 70° and 0.1 mm. pressure to give 47.7 Gm. (73.5%) of product as tiny colorless prisms. A sample was recrystallized twice from Skellysolve B and sublimed for analysis, m.p. 123–125° (capillary with slight decompn.). The infrared spectrum (potassium bromide) showed carbonyl absorption at 1705 cm.$^{-1}$.

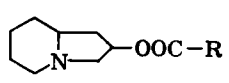
Anal.—Calcd. for $C_8H_{13}NO$: C, 69.03; H, 9.41. Found: C, 68.79; H, 9.40.

The hydrochloride salt melted at 292–293° (decompn. and sublimation, capillary) after several recrystallizations from *iso*propyl alcohol.

Anal.—Calcd. for $C_8H_{14}ClNO$: C, 54.70; H, 8.00. Found: C, 54.91; H, 7.93.

3 - Hydroxy - 1 - azabicyclo[3.3.1]nonane.—

TABLE I.—ESTERS OF 8-HYDROXY-1-AZABICYCLO[4.3.0]NONANE (VII)



Acid (R-COOH) Used for the Esterification	M.p., ° C.	Molecular Formulas	Salt	Analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Activity[a] Muscle Strip[b]	Cat Blood Pressure[c]
Phenyl-2-thienylglycolic[d]	237–238[e]	$C_{20}H_{23}NO_2S$	HCl[f,g]	60.98	60.74	6.14	6.21	100	20
	227–229[e]		CH_3Br[h]	55.75	55.47	5.79	6.03	100	10
Benzilic	217–219	$C_{22}H_{25}NO_3$	HCl[f]	68.12	68.35	6.76	6.68	20	0
	237–238[e]		CH_3Br[f]	61.88	62.11	6.31	6.21	10	0
9-Hydroxyfluorene-9-carboxylic	272–273[e,i]	$C_{22}H_{23}NO_3$	$HCl \cdot {}^1/_2C_2H_5OH$[f,g,k]	67.55	67.66	6.66	6.50	50	1
	249–250[e,i]		CH_3Br[h]	62.15	62.10	5.90	6.00	50	10
Xanthene-9-carboxylic	142–144	$C_{22}H_{23}NO_3$	HCl[l]	68.47	68.15	6.27	6.34	50	0
	234–235[e]		CH_3Br[h]	62.16	62.28	5.90	5.83	100	100
Diphenylacetic	...	$C_{22}H_{25}NO_2$	HCl[m]	...	...	...	...	...	...
	219–220		CH_3Br[f]	64.18	63.91	6.56	6.77	20	0

[a] Expressed as percentage of activity of atropine sulfate. [b] Alleviation due to blocking spasm, induced by 1:10,000,000 dilutions of methacholine, on an isolated guinea pig ileum. [c] Alleviation due to blocking depressor response to 1 mcg./Kg. intravenously of methacholine on the blood pressure of an anesthetized cat. [d] The free base was ether insoluble, so methylene chloride was added following hydrolysis and the salts were prepared in methylene chloride. [e] With decomposition. [f] Recrystallized from ethyl acetate–absolute alcohol–*iso*propyl ether. [g] Slowly soluble in absolute alcohol so the solution was concentrated after filtration. [h] Recrystallized from acetone–absolute alcohol–*iso*propyl ether. [i] Capillary. [j] The melting point is dependent on the rate of heating. The temperature must be raised very slowly over the range 190–250°. [k] Air-dried sample. [l] Recrystallized from ethyl acetate–tetrahydrofuran–*iso*propyl ether. [m] Was an oil which resisted crystallization. Other salts were equally as unsuitable.

A solution of the aminoketone (22.5 Gm., 0.162 mole) in water (50 ml.) was slowly added to a solution of sodium borohydride (1.68 Gm., 0.0445 mole) in water (50 ml.). The solution was allowed to stand at room temperature overnight. Concentrated ammonium hydroxide (50 ml.) (24) was added and the solution allowed to stand for 1.5 hours, after which it was saturated with sodium chloride. The aminoalcohol was extracted with seven 50-ml. portions of benzene. The extracts were dried over anhydrous sodium sulfate and the benzene was removed under slightly reduced pressure to give 20.0 Gm. (87.5%) of product. A sample was recrystallized twice from Skellysolve B and sublimed for analysis at 100° and 0.1 mm. The material was very hygroscopic and a good analysis could not be obtained, m.p. 148–151° (capillary). An infrared spectrum (potassium bromide) showed hydroxyl bands at 3400 cm.$^{-1}$ and 3150 cm.$^{-1}$.

Anal.—Calcd. for $C_8H_{15}NO$: C, 68.04; H, 10.71. Found: C, 67.43; H, 10.69.

8 - Oxo - 1 - azabicyclo[4.3.0]nonane. — Diethyl piperidyl-1,2-diacetate (12) was subjected to the Dieckmann conditions in a manner essentially like that of the 1,3-diacetate except that higher dilutions (2 times the volumes of xylene) were employed. The product was obtained in 60% yield as a colorless liquid that quickly turned to a brown tar upon exposure to air, b.p. 64° (1.25 mm.), n_D^{25} 1.4786 [b.p. 61° (1 mm.) lit. (12)]. The picrate was formed in absolute ethanol and recrystallized from water, m.p. 189° (capillary, with decompn.) [m.p. 187° (decompn.) lit. (12)]. The infrared spectrum (film) showed carbonyl absorption at 1756 cm.$^{-1}$.

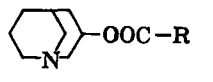
8 - Hydroxy - 1 - azabicyclo[4.3.0]nonane.— The aminoketone was reduced with sodium borohydride as was described for the preparation of 3-hydroxy-1-azabicyclo[3.3.1]nonane, in 89% yield, b.p. 73–74° (0.15 mm.), n_D^{25} 1.4950 [b.p. 95° (14 mm.), lit. (12)].

The picrate was formed in and recrystallized from absolute ethanol, m.p. 175–176° [m.p. 175° (reduction by sodium and alcohol), lit. (12)].

An infrared spectrum (film) showed broad hydroxyl absorption centering at 3400 cm.$^{-1}$.

Methyl Phenyl-2-thienylglycolate.—Methyl phenylglyoxylate (104 Gm., 0.635 mole) [prepared from methyl mandelate as described for the ethyl ester (25) in 97% yield] and anhydrous ether (400 ml.) were placed in a flask equipped with a mechanical stirrer and dropping funnel. The flask was cooled in an ice-salt mixture and a solution of 2-thienyl magnesium bromide [made from 2-bromothiophene (113.8 Gm., 0.7 mole) and magnesium (16.7 Gm., 0.7 Gm. atom)] in anhydrous ether (500 ml.) was siphoned into the dropping funnel and was added to the flask with stirring over a period of 2 hours. The mixture was stirred in the ice-salt bath for an additional hour and then for 3 hours at room temperature. The magnesium addition product was filtered off, washed well with anhydrous ether, and hydrolyzed by stirring it in ether (500 ml.) and cautiously adding a saturated aqueous solution of ammonium chloride (100 ml.) with ice cooling. The ether layer was decanted, washed with water (two 100-ml. portions), 2.5% hydrochloric acid (100 ml.), 5% sodium carbonate solution (two 100-ml. portions), and finally with water (two 100-ml. portions). The ether layer was treated, with charcoal, filtered, and dried over anhydrous sodium sulfate. The ether was removed *in vacuo* and the residue soldified upon scratching and cooling. This was dissolved in 3.5 liters of warm Skellysolve B and carefully cooled to room temperature, additional solvent being added if necessary to prevent oiling. Upon cooling to 10°,

TABLE II.—ESTERS OF 3-HYDROXY-1-AZABICYCLO[3.3.1]NONANE (VI)



Acid (R-COOH) Used for the Esterification	M.p.,° C.	Molecular Formulas	Salt	Analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Activity[a] Muscle Strip[b]	Cat Blood Pressure[c]
Phenyl-2-thienylglycolic	237–238[d,e]	$C_{22}H_{23}NO_3S$	HCl[f]	60.98	61.25	6.14	6.25	100	100
	225–228[d]		CH_3Br[g]	55.75	55.95	5.79	5.89	100	100
Benzilic	230–230.5	$C_{22}H_{25}NO_3$	HCl[f]	68.12	67.97	6.76	6.81	100	100
	225–226		CH_3Br[f]	61.88	61.70	6.31	6.54	100	100
9-Hydroxyfluorene-9-carboxylic	248–251	$C_{22}H_{23}NO_3$	$HCl \cdot {}^1/_2C_2H_5OH$[f,i]	67.55	67.89	6.66	6.93	50	<1
	239–240[d,h]		$CH_3Br.1{-}{}^1/_2C_2H_5OH$[g,i]	60.81	60.81	6.87	7.15	20	<1
Xanthene-9-carboxylic	201–203	$C_{22}H_{23}NO_3$	HCl[f]	68.47	68.24	6.27	6.43	100	10
	216–217		$CH_3Br \cdot {}^1/_2C_2H_5OH$[g,i]	61.67	61.80	6.25	6.11	50	10
Diphenylacetic	208–209	$C_{22}H_{23}NO_3$	HCl[f]	71.05	71.18	7.05	7.13	10	<1
	203–204		CH_3Br[g]	64.18	64.41	6.56	6.73	<10	0

[a] Expressed as a percentage of atropine sulfate. [b] Alleviation due to blocking spasm induced by 1:10,000,000 dilutions of methacholine on an isolated guinea pig ileum. [c] Alleviation due to blocking depressor response to 1 mcg./Kg. intravenously of methacholine on the blood pressure of an anesthetized cat. [d] With decomposition. [e] Capillary. [f] Recrystallized from ethyl acetate–absolute alcohol–*iso*propyl ether. [g] Recrystallized from acetone–absolute alcohol–*iso*propyl ether. [h] Release of ethanol and resolidification at 130–140°. [i] Air-dried sample.

there was deposited 47.5 Gm. of crude ester. Further cooling of the mother liquor gave an additional 17.0 Gm. for a total of 64.7 Gm. (41% yield) of crude ester. This material was recrystallized from warm cyclohexane to give 44.0 Gm. of pure ester, m.p. 62–63°. This material was identical to a sample of the methyl ester of phenyl-2-thienylglycolic acid (21, 26) which had been distilled and had solidified and been recrystallized, m.p. 61.5–62.5°.

Methyl 9-Hydroxyfluorene-9-carboxylate.—9-Hydroxyfluorene-9-carboxylic acid (27, 28) was converted to its methyl ester by the method of Clinton and Laskowski (29), m.p. 165° [m.p. 160° lit. (30)].

1-Azabicyclo[4.3.0]nonan-8-yl Benzilate Hydrochloride and Methobromide.—In a 250-ml. three-neck flask fitted with a mercury sealed stirrer, reflux condenser with drying tube, and a Dean-Stark trap, was placed 8-hydroxy-1-azabicyclo-[4.3.0]nonane (1.0 Gm., 7.1 mmoles), methyl benzilate (1.75 Gm., 7.1 mmoles) and Skellysolve C (freshly distilled from calcium hydride) (75 ml.). A trace of a 46% dispersion of sodium hydride in mineral oil (Metal Hydrides) was added to the refluxing solution with stirring and the mixture refluxed and stirred for 10 hours. Most of the solvent was removed *via* the trap, the mixture cooled, and water (25 ml.) and ether (25 ml.) added with stirring. The organic layer was extracted four times with water (20-ml. portions), and finally dried over anhydrous sodium sulfate. The solvent was removed *in vacuo*, and the residue was taken up in 25 ml. of anhydrous ether. The hydrochloride was prepared by adding ethereal hydrogen chloride. The methobromide was formed by adding methyl bromide, stoppering the flask tightly, and allowing it to remain at room temperature for several days. The salts, in this case and in general, were recrystallized by the solvent mixtures listed in Tables I and II.

1-Azabicyclo[3.3.1]nonan-3-yl Xanthene-9-carboxylate Hydrochloride and Methobromide.—Xanthene-9-carboxylic acid[11] was converted to its acid chloride by refluxing the acid (1.6 Gm., 7.1 mmoles) in thionyl chloride (10 ml.) for 2 hours. The excess thionyl chloride was removed *in vacuo*, and the residue dissolved in dry benzene (15 ml.) and the solvent removed under reduced pressure. A solution was made of freshly sublimed 3-hydroxy-1-azabicyclo[3.3.1]nonane (1.0 Gm., 7.1 mmoles), anhydrous benzene (50 ml.) and triethylamine (1.0 ml.). A solution of the acid chloride in dry benzene (25 ml.) was added dropwise over a period of 1/2 hour, with stirring. The mixture was heated at 70° and stirred for 2 1/2 hours. The mixture was cooled, and the triethylamine hydrochloride filtered off (*ca.* 1 Gm.). The benzene solution of the free base was washed with four 20-ml. portions of water, dried over anhydrous sodium sulfate, and the solvent removed *in vacuo*. The residue was dissolved in 25 ml. of dry ether and the hydrochloride or methobromide formed and recrystallized as outlined in the previous preparation.

[11] The authors wish to express their appreciation to Dr. R. M. Dodson of G. D. Searle and Co. for a generous gift of xanthene-9-carboxylic acid.

SUMMARY

1. The literature preparation of 3-oxo-1-azabicyclo[3.2.2]nonane could not be improved upon. Its isomers and their respective alcohols (3-hydroxy-1-azabicyclo[3.3.1]nonane and 8-hydroxy-1-azabicyclo[4.3.0]nonane) were synthesized in good yield.

2. A series of 19 esters of the named amino-alcohols using five acids was prepared.

3. The pharmacological testing of these esters show several to be potent anticholinergic agents.

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Effects of *dl*-, *d*-, and *l*-Amphetamine on Levarterenol Tachyphylaxis in the Isolated Heart of *Venus mercenaria*

By RAYMOND F. ORZECHOWSKI and DAVID E. MANN, JR.

The comparative potencies of *dl*-, *d*-, and *l*-amphetamine in completely blocking the onset of levarterenol tachyphylaxis (characterized by diminished negative inotropic responses) in the isolated heart of *Venus mercenaria* were as follows: *d*-amphetamine was the most potent; *dl*-amphetamine and *l*-amphetamine were equally active, and less potent than the *d*-isomer. The observance of positive inotropic responses, occurring with higher doses of amphetamine isomers, indicated that both agents may act at the same receptor site. Furthermore, it is speculated that amphetamine may possibly block the inhibitory cardiac sites upon which levarterenol acts, thereby allowing the latter agent to exert a stimulatory action.

THE TERM tachyphylaxis or skeptophylaxis is used to denote a diminution in the response to successive equal doses of a pharmacological agent within a limited time interval. The diminished pressor responses which occur after consecutive doses of ephedrine (1), and the inability of repeated administrations of atropine to depress intestinal motility (2), are well-known examples of this phenomenon.

In 1953 Welsh (3), while evaluating the effects of various drugs on the isolated heart of the quahog—*Venus mercenaria*—observed the development of acute tolerance to repeated administrations of epinephrine. Fujita and Mann (4) reported that tachyphylaxis, characterized by diminished negative inotropic responses, occurred in the *Venus* heart after three or four successive doses of levarterenol. Further studies (5) showed that this response could be partially or completely blocked by ephedrine hydrochloride, *l*-ephedrine being more potent than either the *d*-isomer or the racemic mixture in this respect.

Amphetamine, a sympathomimetic agent chemically related to ephedrine, is believed to exert its peripheral actions in a manner similar to that of ephedrine, *i.e.*, adrenergic blockade (6). Because it had been noted that *l*-amphetamine was more potent than *d*-amphetamine in pressor activity (7), it was the purpose of this investigation to determine whether or not amphetamine would act as ephedrine in this situation and which isomer was the more potent.

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EXPERIMENTAL

The bathing chamber for the isolated heart preparation was constructed of plexiglas and consisted of a section of tubing 4.5 cm. in diameter and 5 cm. in length with a circular base enclosing one end. A hole in the center of the base provided for the insertion of a hypodermic syringe sleeve through which was passed a ground-glass syringe plunger. A small fish hook was fused into the upper end of the plunger. By raising the glass rod vertically until it protruded above the lip of the chamber, the heart could easily be attached to the hook, while lowering the rod immersed the heart in the bathing medium (filtered sea water), thereby minimizing handling and stretching. Inflow and outflow of sea water were accomplished by means of tubes, one of which provided for aeration, inserted through the base of the chamber which was graduated at a volume of 40 ml.

The heart of *Venus mercenaria* was isolated according to the methods of Welsh and Taub (8), and Wait (9). Contractions were recorded on a Livingston long-paper kymograph. The specific gravity of the sea water was maintained at 1.025 and the temperature was kept at 24–26°.

The tabulation of results was done in accordance with the method of Ciuchta and Mann (5). The figures in Table I, representing per cent inhibition, were derived by dividing the lowest amplitude value (in millimeters) recorded within three minutes after the administration of levarterenol, by the amplitude value preceding the administration of the drug. The terms trial I, trial II, and trial III pertain to the three types of treatment that each *Venus* heart underwent. Trial I refers to the administration of three successive doses of levarterenol to establish a tachyphylactic response in a fresh heart; trial II designates that the same heart, after washing, was pretreated with the appropriate isomer and dose of amphetamine sulfate (0.1%) before the subsequent injection of three doses of levarterenol; and trial III refers to the re-establishment of tachyphylaxis by the administration of three successive doses of levarterenol to the heart after washing at the completion of trial II.

Effect of *dl*-Amphetamine Sulfate on Levarterenol Tachyphylaxis.—With the *Venus* heart suspended in the bathing chamber containing 40 ml. of sea

TABLE I.—PER CENT INHIBITION OF THE PREINJECTION AMPLITUDE OF THE HEART OF *Venus mercenaria* AFTER EACH SUCCESSIVE ADMINISTRATION OF 0.40 ML. OF LEVARTERENOL

No.	Trial I — Dose of Levarterenol 1st	2nd	3rd	Trial II — Dose of Levarterenol 1st	2nd	3rd	Trial III — Dose of Levarterenol 1st	2nd	3rd
Trial II Includes *dl*-Amphetamine Sulfate Pretreatment (0.80 mg./40 ml. Sea Water) Prior to Levarterenol									
1	30	21	0	0	12	5	38	30	12
2	0	10	10	0	0	0	35	21	0
3	42	20	0	0	0	0	0	20	59
4	17	8	0	0	0	0	0	18	6
5	49	23	0	0	0	0	38	15	14
6	19	0	0	0	0	0	0	6	0
7	44	16	0	0	0	0	65	5	0
8	15	0	0	0	0	0	7	5	0
9	24	11	0	0	0	0	19	62	46
10	22	19	0	0	0	0	0	0	0
11	40	15	0	0	0	0	23	36	0
12	9	9	0	0	0	0	7	12	0
13	19	33	7	0	0	0	6	9	6
Trial II Includes *d*-Amphetamine Sulfate Pretreatment (0.50 mg./40 ml. Sea Water) Prior to Levarterenol									
1	39	25	0	8	7	0	27	24	0
2	16	7	5	0	0	0	7	9	0
3	100	76	11	0	0	0	5	6	0
4	50	21	0	0	0	0	11	10	0
5	5	15	8	0	0	0	0	0	0
6	35	15	0	0	0	0	7	19	6
7	22	6	0	0	0	0	7	5	0
8	17	14	5	0	0	0	9	5	0
Trial II Includes *l*-Amphetamine Sulfate Pretreatment (0.80 mg./40 ml. Sea Water) Prior to Levarterenol									
1	32	23	11	15	0	0	19	5	0
2	60	27	0	14	0	0	26	13	0
3	69	42	0	0	0	0	44	16	0
4	25	12	0	0	0	0	0	7	0
5	47	0	5	0	0	0	13	5	0
6	16	0	0	0	0	0	6	7	0
7	26	11	0	0	0	0	13	6	0
8	58	28	0	0	0	0	7	10	0
9	25	8	0	0	0	0	8	0	0
10	41	0	0	0	0	0	24	11	0
11	27	25	10	0	0	0	51	41	0
12	19	10	0	0	0	0	0	0	0
13	18	17	0	0	0	0	0	5	0
14	23	10	5	0	0	0	12	37	0
15	34	13	0	0	0	0	25	9	0

water, normal contractions were recorded, whereupon the ventricle was challenged with three 0.40-ml. doses of levarterenol[1] administered at 6-minute intervals to elicit tachyphylaxis. The majority of hearts were unresponsive to the third dose of levarterenol, while several preparations reacted slightly. Nevertheless, the pattern of diminishing responses to the three successive injections indicated that tachyphylaxis had occurred. The concentrations of levarterenol in 40 ml. of sea water after each dose were as follows: 0.4 mg. (1×10^{-5}) after the initial dose, 0.8 mg. (2×10^{-5}) after the second, and 1.2 mg. (3×10^{-5}) after the third. Following the development of the tachyphylactic pattern, the ventricle was allowed to beat for three minutes, then it was washed and rewashed with fresh sea water for one-minute periods. This procedure terminated trial I.

After the wash, the heart was again permitted to beat for approximately three minutes, or until it had resumed normal contractions. It was next treated with a dose of *dl*-amphetamine sulfate (0.40, 0.50, 0.70, or 0.80 mg.) so that when injected into 40 ml. of sea water the concentrations were respectively: 1.0×10^{-5}, 1.25×10^{-5}, 1.75×10^{-5}, or 2.0×10^{-5}. Approximately four minutes later, the heart was treated with three 0.40-ml. doses of levarterenol. This phase of the experiment was referred to as trial II.

Trial III consisted of the administration of three 0.40-ml. doses of levarterenol for the purpose of re-establishing tachyphylaxis in the same heart subsequent to final washing after trial II, and after normal contractions had been obtained.

Pretreatment with the lowest dose (0.4 mg.) of *dl*-amphetamine caused a complete blockage to subsequent doses of levarterenol in 4 out of 10 cases; 0.5 mg. completely blocked only 1 out of 10 cases; 0.7 mg. inhibited tachyphylaxis in 5 out of 8 cases; 0.8 mg. caused a complete blockage of the negative inotropic response in 12 out of 13 determinations (Table I).

Effect of *d*-Amphetamine Sulfate on Levarterenol Tachyphylaxis.—The heart was treated as in the previous part, with the exception of trial II which consisted of pretreatment with a dose of *d*-amphetamine sulfate (0.40, 0.50, 0.60, 0.70, or 0.80 mg.) so that the bath concentrations attained were: 1.0×10^{-5}, 1.25×10^{-5}, 1.50×10^{-5}, 1.75×10^{-5}, or 2.0×10^{-5}.

[1] Levophed bitartrate, 0.1% base, Winthrop Laboratories.

Pretreatment with the 0.40-mg. dose of *d*-amphetamine caused a complete blockage to the negative inotropic response of levarterenol in 3 out of 8 cases. The five remaining hearts were partially blocked as shown by a great diminution of response as compared to trial I. A dose of 0.5 mg. caused 7 out of 8 hearts to be unresponsive to all doses of levarterenol. Nine hearts were treated with either 0.60, 0.70, or 0.80 mg. of *d*-amphetamine (3 at each dose) with the following results: all 9 hearts showed a complete blockage to subsequent doses of levarterenol; in trial III, after thorough washing, 6 of the 9 hearts remained either completely refractory to all doses of levarterenol or partially responsive to only one dose.

Effect of *l*-Amphetamine Sulfate on Levarterenol Tachyphylaxis.—The heart was treated as previously described with the exception of trial II which consisted of pretreatment with a dose of *l*-amphetamine sulfate (0.40, 0.60, 0.70, or 0.80 mg.) to attain bath concentrations of 1.0×10^{-5}, 1.5×10^{-5}, 1.75×10^{-5}, or 2.0×10^{-5}. The 0.40 and 0.60 mg. doses produced complete blockage of levarterenol tachyphylaxis in 1 out of 5 and 1 out of 6 cases, respectively; 2 out of 7 hearts were blocked by 0.70 mg.; and complete blockage of 13 out of 15 hearts occurred at the 0.80 mg. dosage level.

DISCUSSION

The results of trial I have reconfirmed the published observations of Fujita and Mann (4) that tachyphylaxis to the negative inotropic response of levarterenol occurs in the isolated *Venus* heart. In trial II, pretreatment with the isomers of amphetamine sulfate partially or completely blocked this effect in a manner similar to that produced by the isomers of ephedrine hydrochloride (5). The latter study revealed that *l*-ephedrine was the most potent inhibitor of the tachyphylactic response. This investigation has shown that *d*-amphetamine possesses the greatest blocking activity, while *l*-amphetamine and the racemic mixture are approximately equal in their ability to block the tachyphylactic response. The comparative potencies of these isomers are recorded in Table II. From these results, it is reasonable to assume that the spatial configurations of the amphetamine and ephedrine molecules are implicated in the antagonism of levarterenol.

The theory of Gaddum and Kwiatowski (10), which attempted to explain the mechanism of action of ephedrine, has also been applied to amphetamine and offers a possible explanation for the observed effects in the *Venus* heart. Accordingly, amphetamine might exert its antagonistic actions upon the same receptor mechanism which levarterenol activates, *i.e.*, by combining with levarterenol receptors in a manner comparable to substrate competition, thus preventing the onset of acute tolerance. Another similarity between amphetamine and ephedrine was noted particularly at higher dosage levels. When the hearts were pretreated with amphetamine in trial II, positive inotropic effects were frequently seen which were potentiated by subsequent doses of levarterenol, perhaps indicating that both agents may act at mutual receptor sites. Consequently, large doses of amphetamine might block the inhibitory sites and allow levarterenol to produce its positive inotropic action upon excitatory receptors.

The pretreatment of *Venus* hearts with large doses (0.5 mg. plus) of *d*-amphetamine in trial II frequently resulted in a failure to re-establish the tachyphylactic response in trial III, despite several thorough washings. This effect was seldom encountered when *l*-amphetamine was used, but occasionally occurred after large doses of the racemic mixture. It may be due to a binding of the *d*-isomer at the receptor site to prevent the characteristic levarterenol response, a phenomenon which further supports the contention that *d*-amphetamine is the most potent isomer in abolishing levarterenol tachyphylaxis.

SUMMARY

Pretreatment of the isolated *Venus* heart in trial II with various concentrations of the *dl*-, *d*-, and *l*-isomers of amphetamine has indicated that *d*-amphetamine is the most potent inhibitor of levarterenol tachyphylaxis, while *dl*-amphetamine and *l*-amphetamine, although equally active, are less potent than the *d*-isomer.

It is postulated that amphetamine could block levarterenol responses by acting on the same receptor sites which levarterenol affects.

Observance of a positive inotropic effect with higher doses of amphetamine isomers, which is proportional to their relative blocking abilities, and the potentiation of this response with subsequent administrations of levarterenol indicated that both agents may act at the same receptor site. Furthermore, it is presumed that amphetamine may block the inhibitory sites, thereby allowing levarterenol to exert a stimulatory action.

TABLE II.—PERCENTAGE OF *Venus* HEARTS EXHIBITING COMPLETE BLOCKAGE TO ALL ADMINISTRATIONS OF LEVARTERENOL AFTER PRETREATMENT WITH VARIOUS DOSES OF *dl*-, *d*-, AND *l*-AMPHETAMINE SULFATE IN TRIAL II

dl-Amphetamine		*d*-Amphetamine		*l*-Amphetamine	
Dose[a]	% Unresponsive Hearts	Dose[a]	% Unresponsive Hearts	Dose[a]	% Unresponsive Hearts
0.40	40 (4/10)	0.40	38 (3/8)	0.40	20 (1/5)
0.50	10 (1/10)	0.50	88 (7/8)	0.60	17 (1/6)
0.70	63 (5/8)	0.60	100 (3/3)	0.70	29 (2/7)
0.80	92 (12/13)	0.70	100 (3/3)	0.80	87 (13/15)
		0.80	100 (3/3)		

[a] Dose is mg./40 ml. sea water.

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Effect of Ultrasonic Waves on the Stability of Selected Surface-Active Agents, Sulfonamides, and *p*-Aminobenzoic Acid

By G. D. FENN† and P. F. BELCASTRO

Data are presented showing the effect of ultrasonic waves on the stability of certain surface-active agents, sulfonamides, and *p*-aminobenzoic acid. Under the experimental conditions employed, the surface-active agents were stable and the breakdown of the sulfonamides and *p*-aminobenzoic acid was greater than could be accounted for on the basis of peroxide formation.

BEAL and Skauen have reported that the viscosity of several surface-active agents is reduced upon exposure to ultrasonic waves (1). This suggests the possibility of a decrease in molecular weight of these agents as a result of depolymerization or some other type of chemical decomposition. Similar breakdown of large molecules has been reported by several authors (2–5). Araujo has reported the decomposition of procaine penicillin G and sulfathiazole in an ultrasonic field (6). The authors have noted similar decomposition of procaine and buteth-amine hydrochlorides. This decomposition was apparently of an oxidative nature and was prevented by the use of sodium bisulfite as an antioxidant (7). A number of authors have reported the formation of peroxides in aqueous solutions as a result of treatment with ultrasonic waves (8–11). In view of these investigations, it was deemed to be of value to determine if surface-active agents are stable in an ultrasonic field and if peroxide formation is responsible for the breakdown of sulfonamides and similar compounds. This communication reports the effect of ultrasonic waves at a frequency of 400 kilocycles per second upon the stability of selected surface-active agents. This should give an indication as to the advisability of using these agents when preparing emulsions or suspensions by ultrasonics. The amount of peroxides formed as a result of exposure to ultrasonic waves of this frequency was determined. The effect of similar amounts of peroxide alone on the stability of one of the sulfonamides was compared with the effect of ultrasonic waves upon stability. This should indicate if the breakdown due to ultrasonic waves is caused primarily by the formation of peroxides.

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EXPERIMENTAL

Calibration of the Generator.—The ultrasonic generator employed in this study was constructed at Purdue University and was designed for use with a barium titanate transducer. The generator is rated at 250 watts with a variable frequency range. A Hypersonic Transducer, model BU-305,[1] with a focused bowl of barium titanate was employed. The generator was calibrated by determining the wattage produced at various rheostat settings by multiplying the power amplifier voltage and the power amplifier current as shown by appropriate meters on the generator. All of the experiments involving insonation were conducted at a frequency of 400 kilocycles per second and an energy level of approximately 150 watts.

Stability of Selected Surface-Active Agents.—The surface-active agents chosen for this part of the study were Myrj 45,[2] G-2159,[2] Aerosol AY,[3] Tween 80,[2] Span 80,[2] and Triton X-200.[4] Solutions of these surfactants were insonated for extended periods of time. The hydroxyl and saponification

[1] Marketed by Brush Development Co., Cleveland, Ohio.
[2] Marketed by Atlas Powder Co., Inc.
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TABLE I.—SAPONIFICATION AND HYDROXYL VALUES OF SAMPLES OF INSONATED AND NONINSONATED SURFACE-ACTIVE AGENTS

Surface-Active Agent	Hydroxyl Value		Saponification Value	
	Noninsonated Sample	Insonated Sample	Noninsonated Sample	Insonated Sample
Myrj 45	107.2	103.6	93.8	91.8
	102.9	102.3	92.9	92.8
G-2159	19.0	18.9	[a]	[a]
	19.1	19.1		
Aerosol AY	0	0	304.1	303.8
	0	0	305.3	304.2
Tween 80	77.8	77.2	52.3	52.6
	75.3	74.4	52.4	52.7
Span 80	193.3	188.2	156.4	157.3
	190.7	186.0	154.6	156.3
Triton X-200	21.7	19.7	[b]	[b]
	21.1	19.7		

[a] Not determined, due to lack of change in hydroxyl value. [b] Not determined, since compound lacks ester group.

TABLE II.—PER CENT SULFAPYRIDINE REMAINING AFTER ULTRASONIC IRRADIATION

Time, Hr.	Insonated at 25°C.			Controls at 25°C.		
	Sample 1	Sample 2	$\bar{x}$[a]	Sample 1	Sample 2	$\bar{x}$[a]
1	98.7	99.1	98.7	100.0	100.0	100.0
2	98.3	98.3	98.3	100.0	100.0	100.0
3	97.8	98.3	98.0	100.0	100.0	100.0
4	97.4	97.6	97.5	100.0	100.0	100.0
5	97.3	97.8	97.6	100.0	100.0	100.0
6	97.4	97.4	97.4	100.0	100.0	100.0

[a] Arithmetic mean.

values of both insonated and noninsonated samples were determined. Hydroxyl values were determined by the method of Ogg, Porter, and Willits (12), while the saponification values were determined by the official method of the American Oil Chemists' Society (13). A decrease in the saponification value would indicate that ester hydrolysis had occurred while an increased hydroxyl value would indicate either ester or ether hydrolysis, or both, had occurred. The temperature was held at 25° ± 0.5° by circulating cold water through the transducer cooling coil. Table I shows the values obtained for these agents under these conditions.

Stability of Selected Sulfonamides and *p*-Aminobenzoic Acid.—The drugs selected for this portion of the study were sulfanilamide, sulfapyridine sodium, sulfathiazole sodium, and *p*-aminobenzoic acid. Solutions of 0.050 *M* concentration were prepared. To each 0.050 mole of sulfanilamide 0.025 mole of sodium hydroxide was added to give a salt to acid ratio of 1:1, and to each 0.050 mole of *p*-aminobenzoic acid, 0.050 mole of sodium hydroxide was added to form the sodium salt. The solutions were prepared using a pH 10.43 phosphate buffer of such composition that the resulting solutions had ionic strengths of 1.0. The buffer was composed of 11.40 Gm./L. of $Na_3PO_4 \cdot 12\ H_2O$ and 74.28 Gm./L. of $Na_2HPO_4 \cdot 7\ H_2O$. Sample volumes of 30 ml. were exposed to ultrasonic irradiation for time periods of from one to six hours at a temperature of 25° ± 0.5°, maintained by circulating the transducer coupling fluid from a constant temperature bath. Noninsonated control samples were held at the same temperature for equal time periods. For sulfanilamide, control samples were also run at 75° and 100° ± 0.5°. A 25.0-ml. sample of each of the insonated and control samples was then analyzed for remaining drug by the potentiometric titration of LaRocca and Waters (14). The amount of drug present in the samples before treatment was also determined by this method. Table II and Fig. 1 show typical results, while Table III summarizes the findings.

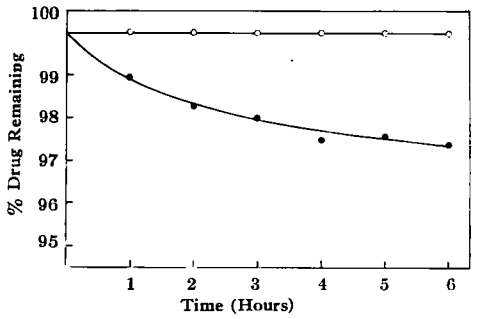

Fig. 1.—Per cent sulfapyridine remaining after ultrasonic irradiation; ●, insonated sample; ○, control sample.

Peroxide Formation by Ultrasonics.—In this portion of the study, samples of a pH 10.43 phosphate buffer with an ionic strength of 1.0 were

TABLE III.—PER CENT OF DRUG REMAINING AFTER ULTRASONIC IRRADIATION

Drug	1 Hr.	2 Hr.	3 Hr.	4 Hr.	5 Hr.	6 Hr.
Sulfanilamide	98.9	97.6	96.7	96.3	95.6	95.4
Sulfapyridine	98.9	98.3	98.0	97.5	97.6	97.4
Sulfathiazole	98.6	98.2	97.8	97.6	97.4	97.4
p-Aminobenzoic acid	99.1	98.5	98.3	97.4	97.1	96.9

insonated at a temperature of 25° ± 0.5° for varying periods of time. The buffer was composed of 11.29 Gm./L. of $Na_3PO_4 \cdot 12H_2O$ and 73.43 Gm./L. of $Na_2HPO_4 \cdot 7\ H_2O$. One-milliliter portions were withdrawn and analyzed for hydrogen peroxide, using a Beckman model B spectrophotometer, by the method of Patrick and Wagner (15). Table IV shows the results of this segment of the study.

TABLE IV.—PEROXIDE FORMATION IN PHOSPHATE BUFFER AS A RESULT OF ULTRASONIC IRRADIATION

Time, Hr.	Absorbance	Moles/L. × 10^5
0.5	0.029	0.15
1.0	0.085	0.4
1.5	0.164	0.8
2.0	0.505	2.5
2.5	1.08	5.3
3.0	1.35	6.6

Effect of Hydrogen Peroxide on the Stability of Sulfanilamide.—Dilutions of varying concentrations of hydrogen peroxide in a pH 10.43 phosphate buffer which, when used to prepare 0.050 *M* sulfanilamide solution, gave an ionic strength of 1.0 were prepared. The hydrogen peroxide concentration ranged from 2×10^{-5} moles/L. to 2×10^{-3} moles/L., or up to about 40 times the amount shown to be produced by ultrasonic waves. These concentrations of hydrogen peroxide had no measurable effect on the stability of sulfanilamide.

DISCUSSION

These data would indicate that the surface-active agents chosen were stable, in terms of ether and ester hydrolysis, to prolonged periods of ultrasonic irradiation. No danger of breakdown would be anticipated if these agents were employed in emulsions or suspensions prepared by ultrasonics.

The stability of the sulfonamides and the *p*-aminobenzoic acid is decreased by exposure to ultrasonic waves. The amount of peroxides formed, however, is not sufficient to account for the observed decomposition of these drugs. It would also appear that very high temperatures would be required to produce a similar degree of decomposition. It seems reasonable to state that other factors must be involved in the ultrasonic decomposition of these drugs. Possible local heating effects or mechanical effects may be partially responsible.

SUMMARY AND CONCLUSIONS

1. The purposes of this study were to determine if selected surface-active agents are subject to ester or ether hydrolysis in an ultrasonic field and if the amount of peroxide formed by ultrasonic irradiation is sufficient to account for the observed decomposition of certain sulfonamides and *p*-aminobenzoic acid.

2. No ester or ether hydrolysis of selected surface-active agents was induced by exposure to ultrasonic waves, as noted by determination of hydroxyl and saponification values.

3. The amount of hydrogen peroxide shown to be formed by ultrasonics is not sufficient to account for the observed decomposition of selected sulfonamides and *p*-aminobenzoic acid. The decreased stability of the sulfonamides and *p*-aminobenzoic acid as well as the amount of hydrogen peroxide formed as a result of ultrasonic irradiation was noted. No decrease in the stability of sulfanilamide with up to 40 times this amount of hydrogen peroxide was noted without insonation.

4. Under the experimental conditions described in this paper, the stability of the surface-active agents investigated was not affected by ultrasonic waves. The effect on the stability of the sulfonamides and *p*-aminobenzoic acid cannot be accounted for by the amounts of hydrogen peroxide shown to be formed by ultrasonic irradiation. Local thermal or mechanical effects, or some other factors must be involved.

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Stability of Ascorbic Acid in a Liquid Multivitamin Emulsion Containing Sodium Fluoride

By JAMES E. TINGSTAD, LEE H. MACDONALD, and PETER D. MEISTER

The kinetics of the thermal oxidative nonphotolytic degradation of ascorbic acid in a liquid multivitamin emulsion containing sodium fluoride have been studied. The vitamin appears to degrade initially by a pseudo zero-order reaction, but other evidence indicates that the true order of the reaction is closer to one. The reaction rate increases rapidly as the pH rises from 3.2 to 3.8, and it is this pH effect which probably causes the reaction to appear zero-order in its initial stages. By use of the Arrhenius equation, data obtained from high temperature studies have been used to predict shelf life at room temperature. Factors affecting the accuracy of the predictions are discussed. Sodium fluoride does not appreciably affect the stability of ascorbic acid in this preparation, but a reaction between sodium fluoride and glass, producing a precipitate (Na_2SiF_6), has been observed.

IN THE development of new pharmaceutical products, investigators are often asked to pass judgment on the shelf life of a product long before complete room temperature stability data are available. It then becomes necessary to predict the room temperature shelf life using data obtained from accelerated kinetic studies of degradation rates at elevated temperatures. The theory and mechanics of such predictions have been well outlined by E. R. Garrett of these laboratories (1–3). In order to make the best use of these methods, it is necessary to give careful consideration to all factors that might affect the accuracy of the predictions.

The present investigation was undertaken to determine the shelf life of a liquid o/w multivitamin emulsion containing, as active ingredients, sodium fluoride and vitamins A, B_6, D, and C. Results of preliminary experiments indicated that the stability of ascorbic acid would be the limiting factor in the shelf life of this product; consequently, only the stability of that vitamin was followed during the accelerated kinetic studies. Although Garrett (2) had already studied the stability of ascorbic acid in this type of system, it had been shown that many additives significantly affect the stability of ascorbic acid (4–6) and that the reactions by which ascorbic acid degrades are many and complex (7–9). Consequently, it was necessary to conduct kinetic studies on the new product before reasonably accurate stability predictions could be made. The effect of pH and sodium fluoride on the ascorbic acid stability and the nature of the reaction between sodium fluoride and glass were also studied to a limited extent.

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EXPERIMENTAL

Composition of the Product.—The product is a water-polyol based o/w emulsion labeled to contain, in each 0.6 ml., 1.105 mg. sodium fluoride, 1.5 mg. vitamin A palmitate in oil, 1.0 mg. pyridoxine hydrochloride U. S. P., 25 mcg. vitamin D_3 in oil, 50 mg. ascorbic acid U.S.P., and various sweetening agents, preservatives, emulsifiers, *etc.* When necessary, the pH of the product is adjusted to the desired level with 0.1 *N* sodium hydroxide. The pH of this product is very erratic and can vary as much as ±0.2 unit from lot to lot and can vary with time within the same lot. This makes it difficult (*a*) to set up experiments studying variables other than pH, and (*b*) to evaluate results of these experiments.

As usual, the overage on the labeled amounts of the vitamins is that required to attain acceptable room temperature shelf life. However, since this report is concerned with the stability of ascorbic acid only, it will suffice to say that the theoretical initial concentration of ascorbic acid is 82.5 mg./0.6 ml. in one formula (Preparation I, pH adjusted to 3.35) and 70.0 mg./0.6 ml. in the other (Preparation II, pH adjusted to 3.20).

Kinetic Studies on the Effect of Temperature on Ascorbic Acid Stability.—After determining (by careful gross visual examination) that the emulsion was stable over the temperature range of the kinetic study, 30 ml. of the preparation was filled into 38-ml. capacity high-density polyethylene bottles; the bottles were then loosely capped and placed in amber 1-lb. ointment jars. When the bottles were tightly capped, the CO_2 pressure buildup, caused by the degradation of ascorbic acid and by the elevated temperatures, ruptured the plastic containers. Glass containers could not be used here because of a reaction between sodium fluoride and glass. The loss of water (through vaporization) from the loosely capped bottles was found to be negligible. Amber jars were used because the product would be stored in opaque cardboard containers; therefore, only the nonphotolytic degradation was of interest.

The jars were then sealed and immersed in constant temperature baths set at 70.0, 60.0, 47.0, and

40.0 (±0.1°). Samples were periodically removed from the baths and immediately refrigerated. Just prior to being assayed (by the U.S.P. iodometric titration method) the samples were allowed to equilibrate at room temperature. Both Preparation I and Preparation II (see previous section) were subjected to this treatment.

Kinetic Studies on the Effect of pH, Initial Ascorbic Acid Concentration and Sodium Fluoride on Ascorbic Acid Stability.—To determine the effect of pH on ascorbic acid stability, one lot of the product was divided into three parts, and the pH of each part was adjusted to a different level (3.15, 3.40, and 3.80).[1] The samples were stored as outlined above, this time at 70.0° only, and the stability was determined in the usual manner.

To help determine the apparent order of the reaction, the effect of initial ascorbic acid concentration on the apparent degradation rate was studied. One lot of the product was made with an initial ascorbic acid concentration of 40.0 mg./0.6 ml. (Preparation III, pH adjusted to 3.50).[2] Again the samples were stored at 70.0° only, and the stability was followed in the usual way.

In order to study the effect of sodium fluoride on the ascorbic acid stability in this product, part of Preparation III was processed without sodium fluoride (Preparation IV, pH adjusted to 3.30).[2] These samples were stored at 70.0° and the stability was determined in the usual manner.

Studies on the Effect of Sodium Fluoride on Glass.—Initially, it was planned to package this product in glass, the standard container for this type of preparation. However, after only 24 hours storage in ordinary soft glass or in U.S.P. Type I glass, the product developed a precipitate. To determine the nature of this material, it was separated from the emulsion by centrifugation and subjected to X-ray diffraction analysis using a General Electric XRD-5 diffraction unit.

To further elucidate the conditions under which this precipitate would form, sodium fluoride A.R. (1.105 mg./0.6 ml.) and ascorbic acid (50.0 mg./0.6 ml.) were dissolved in deionized water; and the pH was adjusted to various levels (2.78, 3.25, 4.10, 4.79, 5.80, and 6.35) with 0.1 *N* sodium hydroxide. The solutions were stored in ordinary soft glass and in high-density polyethylene bottles. They were observed for 1 week at room temperature, after which they were assayed for sodium fluoride. When a precipitate was present in the bottle the clear supernatant was used for the assay sample.

RESULTS AND DISCUSSION

The reactions by which ascorbic acid degrades are many and complex (7–9). It is not the intent here to present a complete discussion of the subject, but rather to briefly summarize the more important details so that the data presented herein will be more meaningful. A more complete account may be found in the literature.

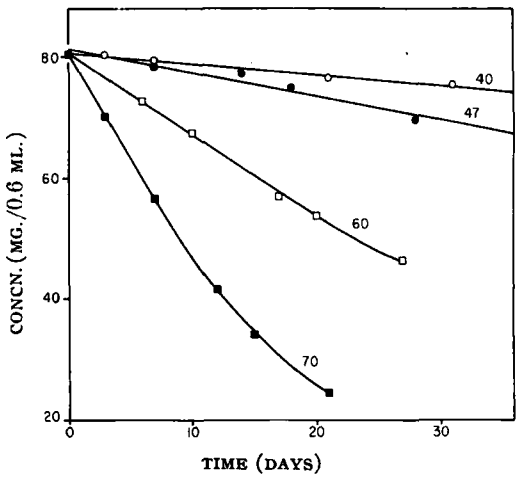

Fig. 1.—Zero-order plots of the thermal oxidative degradation of ascorbic acid in Preparation I; concentration (mg./0.6 ml.) against time (days).

In aqueous solution, ascorbic acid undergoes both a reversible (oxidative) and an irreversible (nonoxidative) degradation. The nonoxidative reaction rate, apparently first-order, decreases with an increase in pH in the 2.2 to 6.0 range; it then increases as the pH rises to 7.0 and above. The products of this anaerobic degradation are furfural, carbon dioxide, and numerous uncharacterized compounds (7). This reaction has not been studied as extensively as the oxidative one.

The oxidative reaction, catalyzed by copper and other trace substances, is 100–1000 times faster than the nonoxidative (7). The aerobic reaction rate increases rapidly as the pH rises from 2.0 to 5.0, then decreases slightly from pH 5.0 to 9.0, then increases rapidly again above pH 9.0. The irregularity of the pH-stability curve seems to be due to the differences in concentrations of the three species of ascorbic acid (undissociated, singly ionized, and doubly ionized) that can exist in aqueous solution at various pH's, and to the different reaction rate constants associated with each of these species (8).

The first step of the oxidation process, in which dehydroascorbic acid is produced, is reversible.[3] However, subsequent oxidation of dehydroascorbic acid is irreversible, and leads to the production of oxalic and threonic acids, carbon dioxide, hydrogen peroxide, and numerous uncharacterized compounds

[1] The pH of the emulsion rises about 0.20 units at room temperature the first one or two days after it is processed; from then on it remains relatively constant at room temperature. The recorded pH's are those measured after the pH has reached this "constant" value. However, at elevated temperatures, the pH continues to rise (0.15 to 0.20 units) during the first part of the kinetic study (how fast it rises depends on the temperature); after that it remains relatively constant. All pH determinations were made on a Beckman Zeromatic pH meter.

[2] In order to eliminate the pH effect on the degradation rate, it was necessary to adjust the pH of these formulas to about the same level as the pH of Preparation I. But since it is extremely difficult to attain the exact desired pH in these systems, there is a measurable pH difference between Preparations I, III, and IV. These differences should be kept in mind when evaluating the data.

[3] The term "reversible," as used here, means that by using appropriate reducing agents, dehydroascorbic acid can be reconverted to ascorbic acid (8). There is a great deal of disagreement as to the optimum pH for the ascorbic acid-dehydroascorbic acid interconversion reaction. Ball (10) says the reaction takes place only below pH 5.0, while Fruton (11) states that it takes place between 5.5 and 7.5. Ghosh and Char (12) have shown that it is possible to reduce dehydroascorbic acid to ascorbic acid in the 2.5 to 7.5 pH range. Levenson (13) says that the irreversible oxidation rate is slowest at pH 3.5. This illustrates that ascorbic acid degradation is extremely complex, and that different results can be obtained by making relatively small changes in the system.

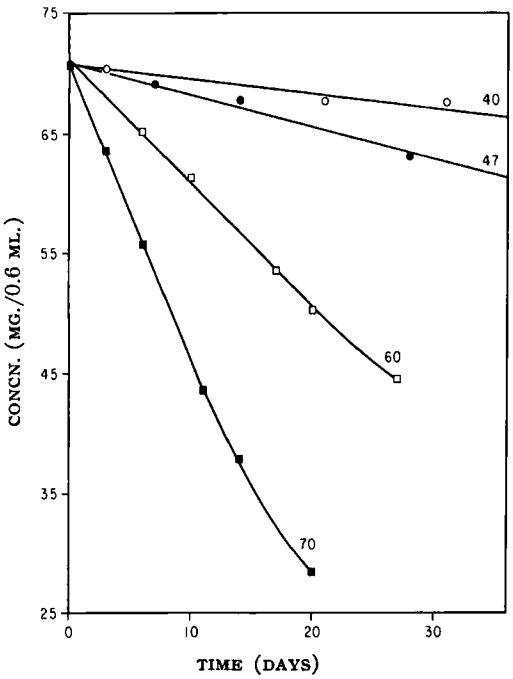

Fig. 2.—Zero-order plots of the thermal oxidative degradation of ascorbic acid in Preparation II; concentration (mg./0.6 ml.) against time (days).

TABLE I.—TABULATION OF SLOPES (k_0),[a] THEIR STANDARD DEVIATIONS (σk_0), AND THEIR 95% CONFIDENCE LIMITS FOR ZERO-ORDER PLOTS

Temp., °C.	k_0 (mg./0.6 ml./day)	σk_0	95% Confidence Limits
Preparation I (pH 3.35 and Initial Ascorbic Acid Concn. 82.5 mg./0.6 ml.)			
70.0	3.411	0.042	2.875–3.946
60.0	1.370	0.024	1.294–1.445
47.0	0.380	0.040	0.254–0.507
40.0	0.176	0.017	0.103–0.250
Preparation II (pH 3.20 and Initial Ascorbic Acid Concn. 70.0 mg./0.6 ml.)			
70.0	2.485	0.029	2.359–2.610
60.0	1.033	0.030	0.939–1.127
47.0	0.271	0.025	0.163–0.379
40.0	0.112	0.021	0.022–0.202

[a] For a plot of concentration *vs.* time, the slope = the zero-order rate constant (k_0).

(8, 9). Some of the oxidation products act as catalysts for the degradation of more ascorbic acid; the drug thus undergoes autoxidation (9).

The literature is in general agreement on the fact that ascorbic acid undergoes apparent first-order degradation in simple drug-buffer systems (8, 9). However, in a more complex pharmaceutical system (containing other vitamins, flavors, sugars, etc.), Garrett (2) found that the degradation of ascorbic acid was pseudo zero-order during the initial phase of the reaction. In the later stages apparent first-order kinetics prevailed.

Effect of Temperature.—The effect of temperature on the degradation rate of ascorbic acid is shown in Figs. 1 and 2 where concentration of ascorbic acid is plotted against time at four different temperatures for Preparations I and II, respectively. The behavior of the points (a straight line can be drawn through the points above 45 mg./0.6 ml.; below that concentration the line is curved) at each temperature indicates that the reaction is pseudo zero-order during its initial stages; however, as the reaction proceeds, first-order kinetics become apparent. These results agree essentially with those of Garrett (2). A summary of the statistical treatment of the zero-order data obtained from Figs. 1 and 2 is given in Table I.

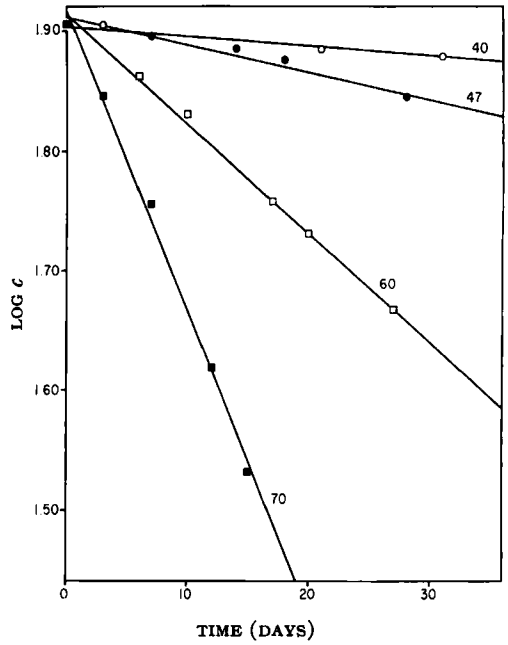

Fig. 3.—First-order plot of the thermal oxidative degradation of ascorbic acid in Preparation I; log concentration (mg./0.6 ml.) against time (days).

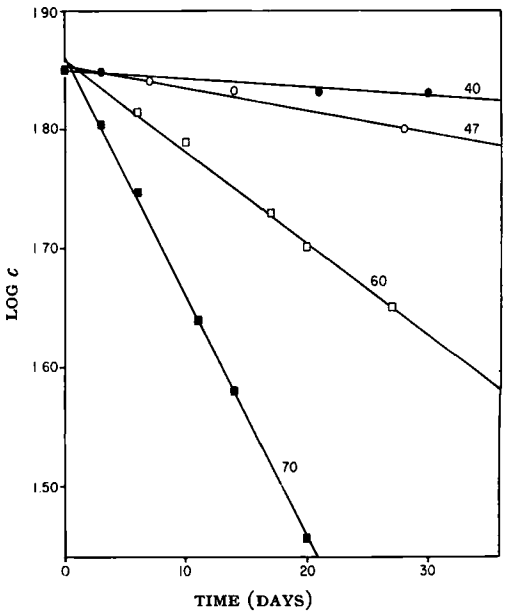

Fig. 4.—First-order plot of the thermal oxidative degradation of ascorbic acid in Preparation II; log concentration (mg./0.6 ml.) against time (days).

TABLE II.—TABULATION OF SLOPES (k_1),[a] THEIR STANDARD DEVIATIONS (σk_1), AND THEIR 95% CONFIDENCE LIMITS FOR FIRST-ORDER PLOTS

Temp., °C.	$k_1 \times 10^2$ (day^{-1})	$\sigma k_1 \times 10^2$	95% Confidence Limits ($\times 10^2$)
Preparation I (pH 3.35 and Initial Ascorbic Acid Concn. 82.5 mg./0.6 ml.)			
70.0	2.532	0.058	2.372–2.692
60.0	0.908	0.025	0.838–0.978
47.0	0.219	0.027	0.135–0.303
40.0	0.098	0.012	0.047–0.148
Preparation II (pH 3.20 and Initial Ascorbic Acid Concn. 70.0 mg./0.6 ml.)			
70.0	2.009	0.042	1.891–2.126
60.0	0.761	0.027	0.686–0.836
47.0	0.176	0.018	0.097–0.255
40.0	0.070	0.013	0.014–0.126

[a] For a plot of logarithm *vs.* time, the slope = k_1 = the first-order rate constant/2.303 = k_1'/2.303.

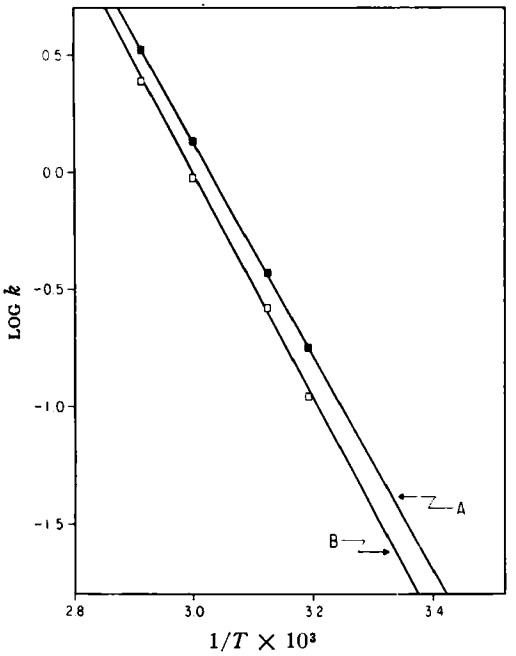

Fig. 5.—Arrhenius plots of log k (zero-order rate constants from Figs. 1 and 2; mg./0.6 ml./day) against the reciprocal of the absolute temperature for Preparation I (plot A) and Preparation II (plot B).

In making predictions of shelf life it is essential to consider the possibility that the reaction might be pseudo first-order from the beginning, and then to consider what the difference is between predictions based on zero-order kinetics and those based on first-order kinetics. Therefore, the logarithm of ascorbic acid concentration was plotted against time (Figs. 3 and 4) for Preparations I and II, respectively. For convenience and ease of calculation, the numerical value of the slopes (k_1) of the lines in Figs. 3 and 4 were used in the Arrhenius plots, calculations of shelf life, etc., rather than the actual first-order rate constant (k_1'). The first-order rate constants could be obtained by plotting logarithm *vs.* time, or by using the following equation: $2.303\ k_1 = k_1'$. A summary of the statistical treatment of these first-order data is given in Table II.

Arrhenius Plots.—In order to best predict the shelf life of this product, Arrhenius plots were made using data obtained from both the zero-order and first-order plots. In Fig. 5 the logarithm of the zero-order rate constants for both preparations (obtained from Figs. 1 and 2) are plotted against the reciprocal of the absolute temperature, while in Fig. 6 the same thing is done for the "first-order" slopes obtained from Figs. 3 and 4. A summary of the statistical treatment of the Arrhenius plot data is given in Table III. It should be noted that the apparent heats of activation for the reaction [these agree very well with those obtained by Garrett (2)] are very similar in all four cases, but that the shelf lives are considerably different, depending on which order is assumed for the reaction. This is due, in part, to the fact that a first-order reaction will slow down as substrate concentration decreases, whereas a zero-order reaction will continue at a constant rate.

Effect of pH.—The effect of pH on the degradation rate of ascorbic acid can be seen in Fig. 7 where a zero-order plot is made for the degradation of ascorbic acid at 70° at three different pH's, and in Fig. 8 where the same data are plotted as first order. The pH dependence of the reaction rate can better be seen in Figs. 9 and 10, where zero-order reaction rates (Fig. 9) and "first-order" slopes (Fig. 10) are plotted against pH. The values for the k's at pH 3.20 and 3.35 were obtained from the slopes of the 70° plots in Figs. 1 and 2. Since

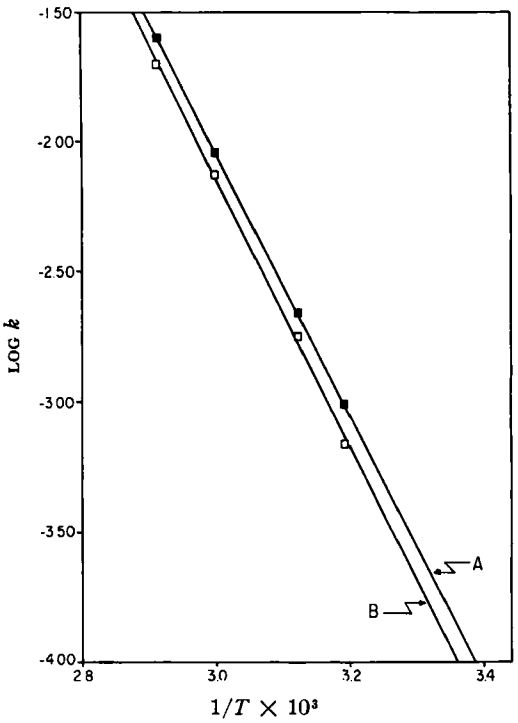

Fig. 6.—Arrhenius plots of log k (slopes of first-order plots from Figs. 3 and 4; day^{-1}) against the reciprocal of the absolute temperature for Preparation I (plot A) and Preparation II (plot B).

TABLE III.—TABULATION OF ARRHENIUS EQUATIONS OF BEST FIT, APPARENT HEATS OF ACTIVATION (ΔH_a), k_0 AND k_1 AT ROOM TEMPERATURE, SHELF LIFE (t_{90})[a] AND 95% CONFIDENCE LIMITS OF SHELF LIFE

Preparation	Equation of Best Fit	ΔH_a (Kcal/mole)	Predicted k_0 and k_1 (25° C.)[b]	t_{90}, Years	95% Confidence Limits of t_{90}
I—Zero-Order	$\log k_o = -4599/T + 13.94$	21.0	0.0326	3.15	2.79–3.56
II—Zero-Order	$\log k_o = -4809/T + 14.43$	22.0	0.0200	3.43	1.90–6.00
I—First-Order	$\log k_1 = -5077/T + 13.20$	23.2	0.000148	4.89	4.29–5.57
II—First-Order	$\log k_1 = -5216/T + 13.52$	23.9	0.000106	4.95	3.16–7.72

[a] The shelf life is defined as the time it takes for the ascorbic acid to degrade to 90% of the labeled potency. Since the labeled potency is 50 mg./0.6 ml., t_{90} for Preparation I is the time it takes for the ascorbic acid concentration to drop from 82.5 mg./0.6 ml. (initial) to 45.0 mg./0.6 ml. (90% of label); and t_{90} for Preparation II is the time it takes for the ascorbic acid concentration to drop from 70.0 mg./0.6 ml. (initial) to 45.0 mg./0.6 ml. (90% of label). [b] Units for k_0 are mg./0.6 ml./day and the units for k_1 are day^{-1}.

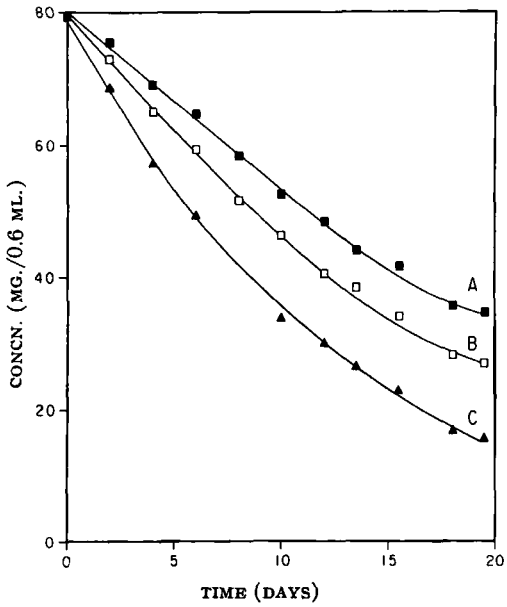

Fig. 7.—Zero-order plot showing the effect of pH on the degradation rate of ascorbic acid; *A*—pH 3.15, *B*—pH 3.40, *C*—pH 3.80; concentration (mg./0.6 ml.) against time (days). Temperature was 70°.

Preparation I and Preparation II have slightly different initial ascorbic acid concentrations, the zero-order reaction rate constants associated with each of these formulas will be slightly different, regardless of pH (see next section). This has not been taken into account in Figs. 9 and 10.

The straight lines in Figs. 9 and 10 are not meant to imply that there is a strict linear relationship between pH and reaction rate in this pH range; rather they are meant to illustrate the significant effect of pH on ascorbic acid stability. Csuros and Petro (8) have represented the pH profile of the reaction as being slightly curved in this pH region. They showed the same significant pH dependence of the reaction rate that is reported here.

Effect of Initial Ascorbic Acid Concentration.—The effect of initial ascorbic acid concentration on its degradation rate is shown in Table IV, where the k's (70°) for Preparation I (82.5 mg./0.6 ml.) are compared with those for Preparation III (40.0 mg./0.6 ml.). The k's for Preparation III were obtained from the *A* plot in Fig. 11 (zero-order) and Fig. 12 (first-order). Note that the pH of Preparation I is lower than that of Preparation III. Considering the pH effects only, a faster rate for Preparation III would be expected. However, the comparison shows that a drop in initial ascorbic acid concentration overcomes the pH effect and slows the rate. The apparent zero-order rate constant is affected much more than is the "first-order" slope; and by using appropriate equations, it can be shown that the true reaction is closer to first-order than to zero-order (14). The cause(s) of the apparent drop in the value of the first-order rate constant when the initial concentration was reduced, even though the pH difference favored a change in the opposite direction, was (were) not investigated.

A possible explanation for the apparent zero-order character of the first part of the reaction is that the initial rise in pH at elevated temperatures (see footnote 3) causes an increase in the reaction rate which offsets the tendency of the first-order reaction to slow down as the substrate concentration decreases. As soon as the pH reaches a constant value, no further deviations from first-order kinetics

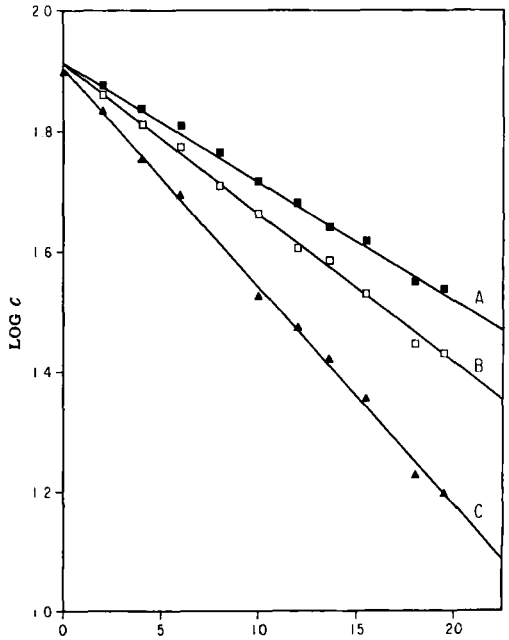

Fig. 8.—First-order plot showing the effect of pH on the degradation rate of ascorbic acid; *A*—pH 3.15, *B*—pH 3.40, *C*—pH 3.80, log concentration (mg./0.6 ml.) against time (days). Temperature was 70°.

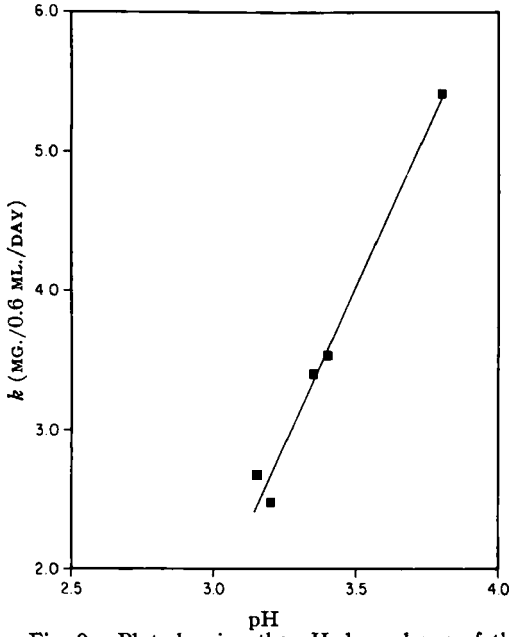

Fig. 9.—Plot showing the pH dependence of the degradation rate; zero-order rate constants (70°; mg./0.6 ml./day) against H.

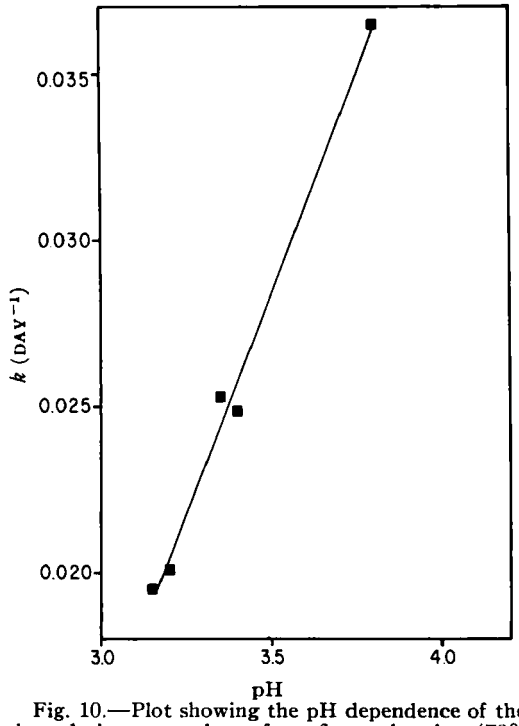

Fig. 10.—Plot showing the pH dependence of the degradation rate; slopes from first-order plots (70°; day^{-1}) against pH.

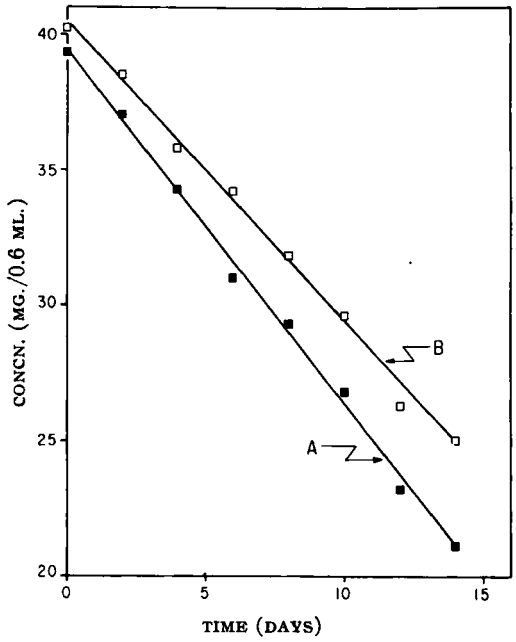

Fig. 11.—Zero-order plot showing the effect of sodium fluoride on ascorbic acid stability at 70°; *A*—Preparation III (with sodium fluoride), *B*—Preparation IV (without sodium fluoride); concentration (mg./0.6 ml.) against time (days).

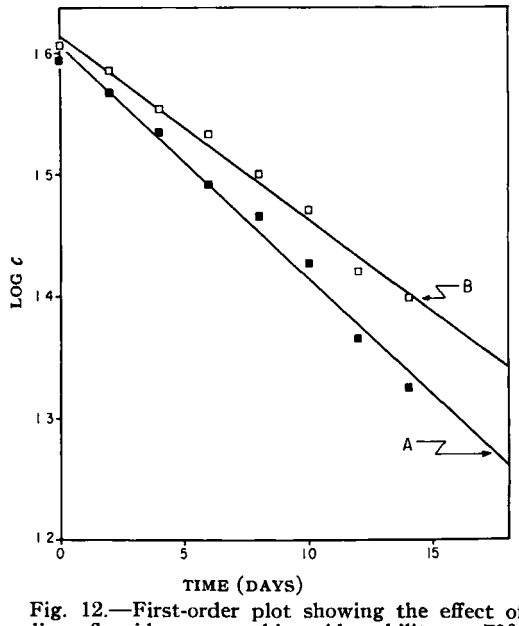

Fig. 12.—First-order plot showing the effect of sodium fluoride on ascorbic acid stability at 70°; *A*—Preparation III (with sodium fluoride), *B*—Preparation IV (without sodium fluoride); log concentration (mg./0.6 ml.) against time (days).

Table IV.—Comparison of Zero-Order Rate Constants (k_0) and "First-Order" Slopes (k_1) for Preparation I (82.5 mg./0.6 ml.) and Preparation III (40.0 mg./0.6 ml.) at 70°C.

Preparation	pH	k_0 (mg./0.6 ml./day)	k_1 (× 10^2 day^{-1})	k_0 (I)/k_0 (III)	k_1 (I)/k_1 (III)
I	3.35	3.411	2.532	2.60	1.31
III	3.50	1.310	1.930		

TABLE V.—COMPARISON OF ZERO-ORDER RATE CONSTANTS (k_0) AND "FIRST-ORDER" SLOPES (k_1) FOR PREPARATION III (WITH SODIUM FLUORIDE) AND PREPARATION IV (WITHOUT SODIUM FLUORIDE)

Preparation	pH	k_0 (mg./0.6 ml./day)	k_1 ($\times 10^2$ day^{-1})	k_0 (III)/k_0 (IV)	k_1 (III)/k_1 (IV)
III	3.5	1.310	1.930	1.17	1.27
IV	3.3	1.120	1.514		

are apparent. The fact that (*a*) the period of pH change corresponds closely with the period during which zero-order kinetics are apparent, and (*b*) the magnitude of the deviations from the first-order character of the reaction is compatible with the magnitude of the pH effect on the degradation rate, supports this explanation.

Effect of Sodium Fluoride.—The effect of sodium fluoride on the stability of ascorbic acid is shown in Figs. 11 and 12. Plot *A* in each figure is the plot of the kinetic data obtained at 70° for Preparation III, while plot *B* is the plot of the kinetic data obtained at 70° for Preparation IV (identical to III except that it contains no fluoride). It can be seen (Table V) that sodium fluoride does not significantly affect the stability of ascorbic acid. The small increase in reaction rate seen with Preparation III can be explained on the basis of its slightly higher pH.

Effect of Sodium Fluoride on Glass.—The precipitate which was isolated from the preparations stored in glass was identified by X-ray diffraction analysis as sodium fluosilicate, Na_2SiF_6 (15). Further studies on the effect of pH on precipitate formation showed that the precipitate forms between pH 2.4 and 4.3 overnight at room temperature, but not between pH 4.8 and 6.5, after 7 days' storage at room temperature. Assays showed no detectable loss of fluoride ion from the system in those preparations in which a precipitate formed. It is possible that a small amount of hydrogen fluoride is liberated at the more acid pH's and that this material attacks the glass. Longer term studies were not carried out because the tests showed that the precipitate forms at the pH's (2.8–3.4) which, because of stability and taste considerations, are optimum for the product. Therefore, it was necessary to package the product in plastic bottles.

Correlation of Predictions with Actual Room Temperature Stability.—The problem in this study was not so much one of predicting shelf life for a product containing a definite amount of ascorbic acid and having a certain pH; rather it was one of deciding what initial ascorbic acid concentration and what pH would allow a shelf life of 2 years for the product. On the basis of the results of this study it was decided to market the product at pH 3.4 or slightly lower with an initial ascorbic acid concentration of 82.5 mg./0.6 ml. These predictions are based on zero-order kinetics because (*a*) the reaction is pseudo zero-order in the concentration range of interest, and (*b*) predictions based on zero-order kinetics give the shortest shelf life; therefore predictions based on zero-order kinetics will err on the safe side, if at all. The first-order treatment of the data has been included for informational purposes. The usefulness of this general approach is demonstrated by the fact that preliminary (6 months) room temperature stability data on the above formulation indicate a shelf life of 2.2–2.8 years.

General Considerations.—An attempt has been made to investigate those problems which could be solved in a reasonable length of time and whose solution might lead to a better product or to a significantly more accurate prediction of shelf life. No attempt has been made to ascertain (*a*) the effect of the polyol concentration and of temperature on the thermodynamic activity of the hydrogen ions and on the dissociation constants of ascorbic acid, (*b*) the effect of ionic strength on the degradation rate, (*c*) the effect of loosening the caps of the polyethylene bottles on the diffusion of oxygen, or (*d*) the effect of temperature on the diffusion rates of oxygen, carbon dioxide, and water vapor through the walls of the polyethylene bottles. These problems were not investigated because (*a*) the system is too complex, and (*b*) the answers to these questions are not likely to lead to a better product or to a significantly more accurate prediction of shelf life. The effect of antioxidants on the ascorbic acid stability was not studied because previous experience had shown that antioxidants are not particularly effective in stabilizing ascorbic acid in this type of system.

SUMMARY

1. The degradation rates of ascorbic acid at elevated temperatures in a liquid, o/w emulsion-type multivitamin preparation have been determined. Arrhenius plots have been made using these kinetic data, and room temperature shelf life has been predicted. Preliminary room temperature studies indicate that these predictions are accurate.

2. The reaction appears to be pseudo zero-order during the initial stages of the reaction; in the latter phases it assumes first-order characteristics. Studies on the effect of initial ascorbic acid concentration on the apparent degradation rate indicate that the true reaction rate is probably closer to first-order than to zero-order.

3. The reaction rate increases rapidly as the pH increases from 3.2 to 3.8. This effect of pH is postulated to be the reason for the zero-order nature of the initial phases of the reaction.

4. Sodium fluoride does not appreciably affect the stability of ascorbic acid in this product.

5. Storing the product in glass results in a precipitate (Na_2SiF_6, sodium fluosilicate) which forms at room temperature within 24 hours between pH 2.4 and 4.3 but not between pH 4.8 and 6.5 after seven days.

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Problems in Chemotaxonomy I

Alkaloids of *Peschiera affinis*

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A phytochemical study of this plant has yielded three indole alkaloids, two of them being of the α-ketoindole type and the third containing a simple nonconjugated indole moiety. One of the α-ketoindole alkaloids has been identified as vobasine, whose recently described structure is found compatible with NMR spectral data. The isolation procedures are described for the second α-ketoindole alkaloid, affinine, and for the indole alkaloid, affinisine.

THE HISTORY of the genus *Tabernaemontana* L. (family *Apocynaceae*) is exceedingly complex. At various times, botanists have split other genera from it (*e.g.*, *Voacanga*, *Ervatamia*, *Gabunia*, *Conopharyngia*) leaving a residue of synonymy of genera and/or species within the group. With regard to South American representatives, botanical opinion concerning their classification has varied widely. Markgraf has separated and readjusted the genus *Tabernaemontana* L. into nine genera, preserving that name for species found in the Antilles, Central America, and parts of northwestern South America, while maintaining *Peschiera* A. DC. for those found in Brazil (1). Woodson does not share this opinion (2).

There is reason to believe that even in closely related genera sufficient differences in alkaloid composition exist to serve as a basis for chemotaxonomic distinctions. In Farnsworth's excellent review of the periwinkles (3) one notes that of the 48 characterized alkaloids isolated from *Vinca* and *Catharanthus* species, only one, akuammine (vincamajoridine) is common to both genera. Obviously, it will be necessary to know more about the actual structures before detailed comparisons can be made between the chemical types found in each genus. As of now, however, it can be stated that the dimeric C_{46} bases have been found only in the genus *Catharanthus*.

From the chemical point of view, the genera in the subtribe Tabernaemontainae have been found to contain two major structural types of alkaloids: the predominant ibogaine, I, type (4, 5) and the tabernaemontanine,[1] II, type (6, 7).

In the course of a broad screening program, about thirty plants which were identified as either *Tabernaemontana* or *Peschiera* species by South American botanists have been encountered. In order to detect any chemical differences which may exist between, and perhaps thus differentiate, these closely related genera, we have chosen a number of them for detailed study. The first of these, reported here, is *Peschiera affinis* (Meull.-Arg.) Miers.

DISCUSSION

A 5.8-Kg. sample of *P. affinis*[2] collected in northeastern Brazil was extracted with alcohol until removal of alkaloidal material was complete (Mayer's reagent). The extract was concentrated under reduced pressure and the residue was treated with dilute phosphoric acid. The filtered, aqueous

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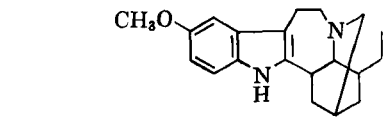

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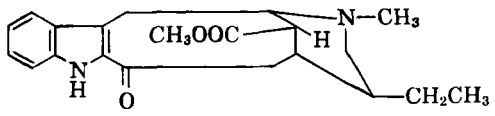
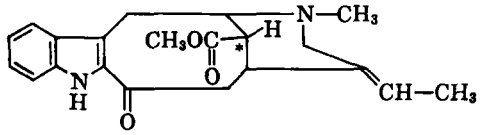


II

solution (pH 1) was subjected to continuous exhaustive extraction with ether. The pH of the aqueous phase was adjusted to 7 and exhaustive extraction with ether was repeated. Finally, extraction with chloroform, after further adjustment of pH to 10, removed residual non-quaternary alkaloidal materials. The quaternary constituents of the aqueous solution were investigated separately and will be the subject of a future communication. The bulk of the crude alkaloids was obtained in the ether extract made at pH 7. Much smaller amounts were found in the chloroform fraction, and the ether extract made at pH 1 was devoid of materials giving a positive alkaloid test.

The ether (pH 7) fraction was chromatographed on 100–200 mesh Florisil. Elution with chloroform gave (in the early fractions) a viscous oil which yielded a crystalline hydrochloride on treatment with ethyl acetate-ethereal hydrogen chloride solution. The regenerated base, obtained in *ca.* 0.45% overall yield, was found identical to the known alkaloid vobasine, III (6, 9, 10), by a mixed melting point determination, and by a comparison of its infrared spectrum and X-ray diffraction pattern with those of an authentic sample.[3]

The nuclear magnetic resonance spectrum[4] of vobasine was found to be in good accord with the elegant structure III, arrived at by Renner and Prins (6) on the basis of chemical as well as electronic and vibrational spectral results. The NMR data are described and interpreted as



III

δ (11)	Assignment
1.70	Vinyl-CH_3 split into doublet by adjacent proton
2.58, 2.65	Ester OCH_3 and NCH_3
3.50	A two-proton singlet from methylene adjacent to the basic nitrogen
5.43	Vinyl proton split into quartet by adjacent methyl
~7.3	Aromatic protons
9.37	N—H hydrogen bonded to carbonyl

[3] We are grateful to Dr. U. Renner, Geigy A. G., Basel, Switzerland, for an authentic sample of vobasine.

[4] The NMR spectrum was determined and interpreted by Dr. G. O. Dudek, Chemistry Department, Harvard University.

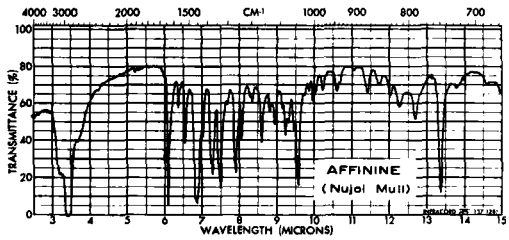


Fig. 1

Some commentary is required with regard to the signals assigned to methoxyl and tertiary aliphatic N-methyl. The expected shifts (as compared to tetramethyl silane) for the two groups are *ca.* 3.7–3.8 and 2.2–2.6 p.p.m., respectively (11, 12, 13). Thus, either signal may be assigned to the N-methyl. The striking shift of O-methyl to higher fields can be explained on the basis of the stereochemistry at C* (as written in III) by an interaction with the diamagnetic portion of the induced field from the aromatic π electrons leading to the observed increased shielding effects (14). If the alternative stereochemistry does in fact

obtain, an $N:\rightarrow C(=O)-OCH_3$ interaction would be expected which also leads to increased shielding of methoxyl, but this time accompanied by a diminished shielding of the N-methyl. In either event there will be a small (~0.2–0.3 p.p.m.) diamagnetic effect from the adjacent double bond on the latter grouping. This situation is also compatible with the observed signals. The resolution of this point is presently under investigation and will be the subject of a future communication.

The above bands represent the majority of the substituents of the molecule and are located and spin-spin coupled in conformity with III. The remaining signals from protons located in the fused ring system cannot be assigned with any certainty as the expected signals cover a small portion of the spectrum. In addition, both their position and spin-spin couplings are dependent upon ring geometry and conformation.

Further elution of the column yielded two new alkaloids, affinisine, $C_{19}H_{24}N_2O$, (5.5 Gm., 0.095%, isolated as hydrochloride) and affinine, $C_{20}H_{24}N_2O_2$ (0.85 Gm.[5]).

Affinisine has an ultraviolet absorption spectrum indicating the presence of a simple nonconjugated indole. Its infrared spectrum (Nujol mull) shows no bands in the 5.5 to 6.5 μ region corresponding to a carbonyl function and did not afford a decision as to the presence or absence of a hydroxyl group on the basis of the 3 μ region. A negative methoxyl determination requires that the single oxygen be present as in either an internal ether or a hydrogen bonded hydroxyl group.

The ultraviolet spectrum of affinine in 95% ethanol indicates the presence of an α-keto indole moiety similar to that found in tabernaemontanine, II, vobasine, III, dregamine (7, 10, 15), voacafrine (16), voacafricine (16), and perivine (17). The infrared spectra of these alkaloids are all reported to

[5] Additional affinine was isolated from the chloroform extract at pH 10 to bring the total yield to *ca.* 0.07%.

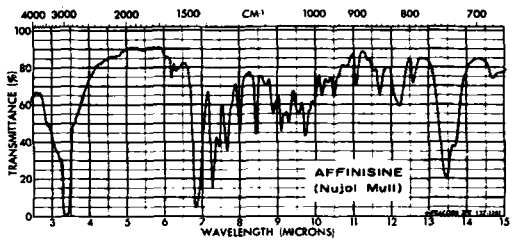


Fig. 2

contain a band at *ca.* 6.06–6.08 μ ascribable to the keto indole grouping, accompanied by a band at 5.79–5.80 μ, probably due to an ester grouping. The infrared spectrum of affinine contains only one band, at 6.08 μ, making it the first example of an alkaloid of this type with a single carbonyl functionality. Its empirical formula, $C_{20}H_{24}N_2O_2$, corresponds to a des-carbomethoxylated voacafrine ($C_{22}H_{26}N_2O_4$) to which it may be directly related.

Isolation of two α-keto indole alkaloids as well as a third containing a simple indole chromophore is in good accord with the predicted general botanical-chemical relationship with *Tabernaemontana*, *Ervatamia*, *Voacanga*, etc. The absence of any of the known alkaloids of the ibogaine type is suggestive of differences between *Peschiera* and the other genera. However, the structure determination of affinisine as well as additional chemical studies in this area are necessary before a firm generalization can be made.

PHARMACOLOGY OF VOBASINE, AFFININE, AND AFFINISINE

Vobasine hydrochloride exhibited weak, but significant, central nervous system depression and ataxia after oral doses of 200 mg./Kg. in mice. Doses of 300 mg./Kg. caused lacrimation, mydriasis, and respiratory depression as well as a progressive increase in the central depressant activity. This compound failed to produce overt effects in cats receiving doses of 15 mg./Kg. intraperitoneally, but was found to produce vocalization, mydriasis, and short-lived, tonic-clonic convulsions following doses of 35 mg./Kg. intraperitoneally. When injected intravenously into the ether-chloralose anesthetized cat at doses ranging from 0.5 to 5.0 mg./Kg. it produced transient depressor activity. A dose of 10 mg./Kg. proved lethal as a result of respiratory depression. No significant changes were observed in the standard test agents (epinephrine, norepinephrine, DMPP, or furfuryl trimethyl ammonium iodide) which measure alterations in the autonomic nervous system after drug treatment. No diuretic activity was observed in the saline hydrated rat receiving doses of 25 mg./Kg. of vobasine orally. Utilizing a modification of the method of Randall and Selitto (18) as presumptive evidence for anti-inflammatory action, we found that vobasine in doses of 25 mg./Kg. orally produced very weak analgesia and moderate antipyretic action, but failed to reduce edema in the rat paw. In the rat, doses of 100 mg./Kg. orally produced signs of toxicity.

Affinine produced gross behavioral changes in the mouse similar to those observed with vobasine. Orally, doses of 200 mg./Kg. caused only slight CNS depressant action, whereas doses of 300 mg./Kg. produced delayed intention tremors, marked CNS depressant activity, ataxia, hypothermia, and bradypnea. No overt effects were observed in the unanesthetized cat receiving 25 mg./Kg. of the compound intraperitoneally. In the anesthetized cat, affinine produced only transient lowering of the mean arterial blood pressure after a cumulative dose as high as 19 mg./Kg. intravenously. Some evidence of toxicity was uncovered since bradycardia, respiratory depression, and cardiac arrhythmias were observed as the dose levels were increased. Affinine was practically inactive when tested for analgetic, antipyretic, or antiedema activity in the Randall and Selitto anti-inflammatory test. No diuretic action was produced in the saline hydrated rat.

Affinisine produced central nervous system depression, lacrimation, and tremors after doses of 50 mg./Kg. orally in the mouse. However, no side effects were observed in the unanesthetized cat following a dose of 10 mg./Kg. intraperitoneally. In the ether-chloralose anesthetized cat, affinisine caused respiratory toxicity after a dose of 1.0 mg./Kg. and proved lethal after an acute dose of 10 mg./Kg. intravenously. Affinisine possesses moderate analgetic activity in the rat after doses as low as 25 mg./Kg. orally. On the other hand, this activity was not accompanied by an effect on skin temperature or on edema. There was no evidence of diuretic activity when affinisine was tested in doses of 25 mg./Kg. orally in the saline-loaded rat.

EXPERIMENTAL[6, 7]

Isolation

The ground whole plant of *P. affinis* (5.8 Kg.) was extracted in a Soxhlet type apparatus with hot 95% ethanol until the marc gave a negative alkaloid test (Mayer's reagent). The extract was concentrated *in vacuo* to a syrupy consistency and the residual solvent was removed at 25° in a stream of air.

The residue was extracted with phosphoric acid (0.2 *M*, total of 14 L.) and the resultant mixture was filtered with Celite. The aqueous solution was treated with ether in a liquid-liquid continuous extractor to yield *ca.* 7 Gm. of nonalkaloidal material. An aliquot (*ca.* two-thirds) of the aqueous layer was neutralized (pH 7) with concentrated sodium bicarbonate solution and extracted to completion with ether. The organic layer yielded 43 Gm. of alkaloid as a brown, crispy foam, which was further processed as described below. The aqueous phase was made alkaline with ammonia and extracted with chloroform to yield an additional 2.3 Gm. of an alkaloidal precipitate and *ca.* 3.5 Gm. of viscous bases.

Forty grams of the alkaloids, extracted at pH 7,

[6] Melting points were determined on a Thomas-Hoover Uni-Melt apparatus. Ultraviolet absorption spectra were obtained using a Cary model 14 spectrometer, and infrared spectra were obtained using a Perkin-Elmer model 173B.

[7] We are indebted to Mr. Irving Eisdorfer of our laboratories for the following paper chromatographic procedure used throughout this study. The mixture *iso*-amyl alcohol: *tert*-amyl alcohol:water:88% formic acid–5:5:10:2 was shaken well. The separated lower layer was used as standing phase and the upper layer as the moving phase on 3 mm. Whatman paper (12 in. × 18 in. sheet) arranged for circular development. The spray reagent used was potassium iodoplatinate.

was dissolved in chloroform and chromatographed on a Florisil column (1 Kg. adsorbent, 3 in. diameter) made up with chloroform. The initial chloroform eluate (*ca.* 3 L.) on combination and evaporation yielded a pale yellow, viscous liquid. Treatment with ethyl acetate-ethereal hydrogen chloride yielded a white crystalline product (17.9 Gm.). Recrystallization from ethyl acetate-methanol gave an analytical sample, m.p. 247° (decompn.), R_f 0.65.

Anal.—Calcd. for $C_{21}H_{24}N_2O_3 \cdot HCl$: C, 64.84; H, 6.48; N, 7.21; Cl, 9.12; O—CH_3, 8.05; N—CH_3 (calcd. CH_3), 3.89; C—CH_3, 6.98. Found: C, 64.42; H, 6.37; N, 7.10; Cl, 8.84, O—CH_3, 8.06; N—CH_3, 3.48; C—CH_3, 5.01.

The free base was obtained by treatment of the hydrochloride with ammoniacal chloroform and isolation of the alkaloid in the usual manner. Recrystallization from ether yielded an analytical sample, m.p. 112.5–115°.

Anal.—Calcd. for $C_{21}H_{24}N_2O_3$: C, 71.57; H, 6.86; N, 7.85. Found: C, 71.32; H, 6.98; N, 7.94.

A mixed melting point determination with authentic vobasine,[3] m.p. 112.5–115° gave m.p. 112.5–115°. The infrared spectra (Nujol mull) of this base and of authentic vobasine were essentially identical and their X-ray diffraction patterns were superimposable.

Affinisine

Further elution with chloroform (9 L.) gave an eluate which on combination and evaporation yielded a viscous oil. On treatment with ethereal hydrogen chloride-ethyl acetate a white crystalline product (5.55 Gm.) was obtained. Recrystallization from methanol gave fine needles, m.p. 287° (decompn.) $[\alpha]_D^{25}$ +40.3 (C = 0.5, MeOH), R_f 0.76.

Anal.—Calcd. for $C_{19}H_{24}N_2O \cdot HCl$: C, 68.57; H, 7.52; N, 8.42; Cl, 10.65; O—CH_3, 9.31; N—CH_3 (calcd. CH_3), 4.50; C—CH_3, 8.11. Found: C, 68.78; H, 7.36; N, 8.30; Cl, 10.44; O—CH_3, negligible; N—CH_3, 3.53; C—CH_3, 3.58. $\lambda_{max.}^{EtOH}$ 224 mμ (log ε 4.65), 282 mμ (log ε 3.92), 292 mμ (log ε 3.82).

The free base was obtained in the usual manner and was recrystallized from methylene chloride-petroleum ether, m.p. 115–118° (insertion at 100°).

Anal.—Calcd. for $C_{19}H_{24}N_2O$: C, 76.99; H, 7.53; N, 9.45. Found: C, 77.07; H, 7.79; N, 9.05, negligible O—CH_3.

Affinine

Elution of the chromatographic column with 15% methanol in chloroform (2 L.) yielded a viscous oil which readily crystallized (0.85 Gm.) from ethyl acetate. Recrystallization from methanol-ethyl acetate yielded an analytical sample as a white crystalline powder, m.p. 265° (decompn.), R_f 0.60.

Anal.—Calcd. for $C_{20}H_{24}N_2O_2$: C, 74.04; H, 7.46; N, 8.64. Found: C, 74.19; H, 7.49; N, 8.64. $\lambda_{max.}^{EtOH}$ 238 mμ (log ε 4.18), 318 mμ (log ε 4.34).

The hydrochloride salt was prepared by solution in methanol, addition of ethereal hydrogen chloride, and removal of solvent *in vacuo*. An analytical sample, m.p. 267–269° (decompn.) was obtained by recrystallization from methanol-ethyl acetate $[\alpha]_D^{25}$ −105.4 (C = 0.5, MeOH).

Anal.—Calcd. for $C_{20}H_{24}N_2O_2 \cdot HCl$: C, 66.56; H, 6.98; N, 7.76. Found: C, 66.21; H, 7.03; N, 7.88, negligible O—CH_3.

Further elution of the column with 15% methanol in chloroform (5 L.) yielded only dark viscous oils (9.9 Gm.), which were not further characterized.

Infrared examination of the alkaloidal precipitate obtained at pH 10 with chloroform showed it to be essentially pure affinine.

No additional non-quaternary crystalline compounds were obtained upon chromatography of any other materials from this plant.

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A Rheological Study of the Aging of Veegum Suspensions

By JOHN H. WOOD, GREGORY CATACALOS, and S. V. LIEBERMAN†

Veegum HV and Veegum F suspensions, 2 per cent by weight in water, were found to be pseudoplastic at all ages for all temperatures of preparation and storage. The study is reported over the time range of a few hours to several months for temperatures from 6 to 88°. The logarithm of the apparent viscosity at any shear rate was found to be a linear function of the logarithm of time, and also to be a function of the reciprocal of temperature, obeying an Arrhenius type relation. The apparent activation energy for Veegum hydration was found to be 8000 calories per mole. A general equation is empirically derived to relate viscosity at any given shear rate with the temperature of storage and the age of the suspension.

TODAY VEEGUM[1] has become one of the more commonly used clay-type suspending agents for the whole range of pharmaceutical and cosmetic formulation. Probably the most popular is Veegum HV but a processed variant, Veegum F, has also a wide acceptability in certain special applications. Veegum is a complex magnesium aluminum silicate containing small amounts of other metal oxides. In composition it is intermediate between aluminum silicate (montmorillonite or bentonite) and magnesium silicate (saponite); these are platy and rod-like, respectively (1). The Veegum lattice tends to the rod form but is alleged to be the form most readily swollen by water. The extensive literature on application of this colloid as a thickening, suspending, or stabilizing agent in diverse products for human use, both internal and external, has been summarized elsewhere (2).

Veegum hydrates more completely as temperature is elevated and held during the suspension period. This hydration leads to the growth of a strong gel, the gel strength increasing with the increased temperature used to make it. Presumably this is due to the particles tending to disperse down to their ultimate structure as water penetrates and swells the lattice. Eventually the medium is a semicontinuous silicate-water complex.

Only limited literature (1, 3, 4) discusses the properties of Veegum suspensions in water alone. The temperature used to make the gel affects the resultant properties (2). The time of hydration for a given procedure as well as the method of mixing have been shown to be critical to the properties of the resultant gel (3, 4). In the most recent study (4), "the processed suspensions were stored . . . for a minimum of sixty days to allow system equilibrium to be established."

It was, therefore, of interest to consider in a single study, for a given arbitrary method of preparation, the rheological aging characteristics of Veegum suspensions prepared and stored at different temperatures.

EXPERIMENTAL

Sample Preparation.—All suspensions, 2% by weight, were prepared in a Waring Blendor, model CB-3, in lots of 1 Kg. by the addition of the Veegum to the water. Mixing was 2 minutes at the slowest of the 5 speeds, followed by 5 minutes at the highest speed. The procedure chosen was quite arbitrary and no attempt was made to evaluate effects introduced by deviations from it.

The following temperatures were used for hydration and subsequent storage: Run 1, room temperature, 40, 73, and 88°; Run 2, 6.5°, room temperature, 32, 40, 50, and 60°. The initial water temperature was chosen so that the final hydration temperature did not exceed the values given. Suspensions were made for both Veegum HV and Veegum F. All samples, except for 6.5°, room temperature, and 40° were stored in forced draft air ovens. The 6.5° sample was in a laboratory refrigerator, the room temperature was ambient, and the 40° in a constant temperature room.

Rheological Examination.—All evaluations were made at 25° immediately upon attainment of that temperature. The rheometer was the modified Brookfield LVT described elsewhere (5), permitting shear rates up to 20 sec.$^{-1}$. Only in the last stages of the study was the similarly modified RVT available. Because of its higher speeds, shear rates to 45 sec.$^{-1}$ were available for samples in the appropriate viscosity range. All rheological measurements were made on aliquots removed as required from bulk storage. No samples were ever reused.

The time intervals for measurement were chosen as convenient for measurement rather than at fixed intervals since the logarithm of time plot was being used to follow changes. Thus samples are compared at corresponding times by graphical interpolation. All interpolations were from plots with point to point joining, rather than from smoothed lines.

Since the sample rheology was time dependent, it

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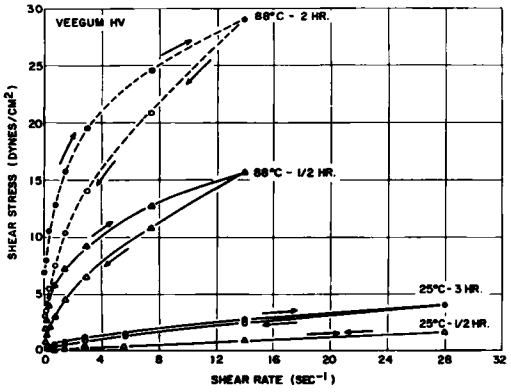

Fig. 1.—Typical rheograms for newly formed Veegum HV mucilages.

was not possible to replicate rheograms in the early hours. At later times, since each rheogram fitted satisfactorily into the pattern of all those preceding it, no attempt was made at replication, except for the example shown in Fig. 2. This is at a period of maximum thixotropy and hence of greatest difficulty in duplication.

With the modified Brookfield in use here, the time constant is such that, in viscous Newtonian systems at very low shear rates, several revolutions of the bob are necessary to reach the steady state reading. Generally, two or three rotations at the low speeds were all that was necessary to attain maximum torque in this study. Immediately upon attaining this value, the speed was increased to the next increment, thus minimizing thixotropic breakdown during the ascending curve. This is the accepted best procedure for systems of this type. All points shown in this study are from the ascending shears only. In Figs. 1 and 2 are shown the gambit of typical rheograms in the initial hours after preparation and after extensive aging, respectively.

Intrinsic Viscosity Comparison.—Suspensions of Veegum HV in 0.5*M* sodium chloride were made for 11 concentrations of Veegum in the range 0.01 to 0.4 wt. per cent, using the Waring Blendor as before. These suspensions were made at room temperature, split into two fractions, and were held in tightly sealed bottles at room temperature and 95° for 1 week. A further 2 days were allowed for the 95° sample to cool. The bottles were checked for any weight loss. Viscosity measurements at 25° were run both for ascending and descending speeds using the "O" cup and bob of the Epprecht (Drage) viscometer. The measuring system is ideal for intrinsic viscosity studies on systems where the normal capillary methods are subject to blockage (6). Shear readings were taken over the full range of speeds for both ascending and descending rates of shear. Limiting slopes of shear stress against shear rate extrapolated to zero shear rate were taken as the viscosity of the sample.

RESULTS AND DISCUSSION

General Rheology and Aging Characteristics.—As initially prepared, the suspensions were very thin. They showed limited thixotropy and were typically pseudoplastic in both ascending and descending shear curves (see Fig. 1). As aging continued, the yield value character already evident in Fig. 1 became more pronounced; both static and dynamic yield values are evident in Fig. 2. This phenomena and this typical rheogram shape has been discussed elsewhere (7). It should be noted that extensive aging introduced a mild rheopectic behavior into the low shear rheogram replacing the earlier minor thixotropy. More severe shear would have probably shown sufficient thixotropy to mask the very low shear rheopexy. Veegum F exhibited similar properties throughout to Veegum HV but, as is clearly evident from Fig. 2, did not show anywhere near as much build-up of plastic body, by a factor of over 10 in viscosity or shear stress units. This difference is also exhibited in comparing the 10 sec.$^{-1}$ curve for HV in Fig. 4 with the 50° isotherm for F in Fig. 5.

In the shear rate permitted by the modified Brookfield LVT rheometer, approximately 0.1 to 20 sec.$^{-1}$, all the 2% Veegum suspensions behaved in this study as almost ideal pseudoplastic systems. Thus a plot of the log of the shear stress, T, against the log of the shear rate, D (log T *vs.* log D), gave essentially straight line plots, an example of which is given in Fig. 3. The slope, or Power law index, ranged from 0.70 to 0.33 for the time interval shown in Fig. 1. Characteristics to be noted are the increase in shear stress required with time, and the marked decrease in the slope of the line with increasing sample age. It is more convenient to deal with apparent viscosity, η_D, defined as T/D, for various given values of the shear rate. The resultant plot, Fig. 4, shows clearly that with age the apparent viscosity becomes increasingly dependent on the shear rate of measurement used.

Recently the application of log viscosity-log time plots for the following of the aging of systems which develop viscous body with time was discussed (8) for use with lotion products. This type of plot was applicable for all the samples studies. Within a set

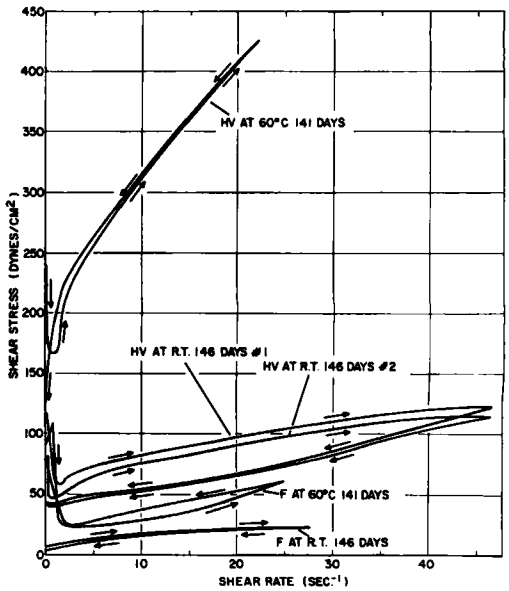

Fig. 2.—Typical rheograms for aged Veegum suspensions.

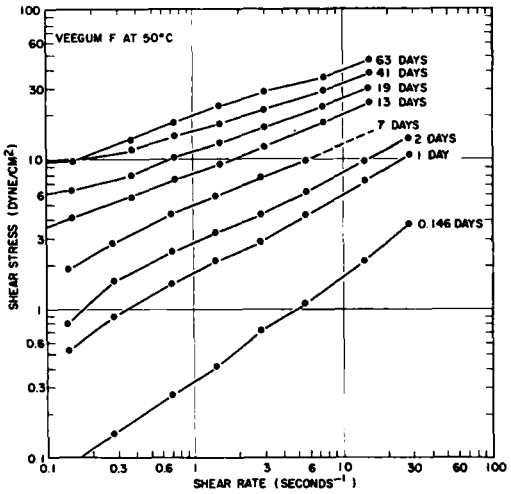

Fig. 3.—Shear stress *vs.* shear rate curves for a 2% Veegum F suspension prepared and held at 50° for varying periods of time.

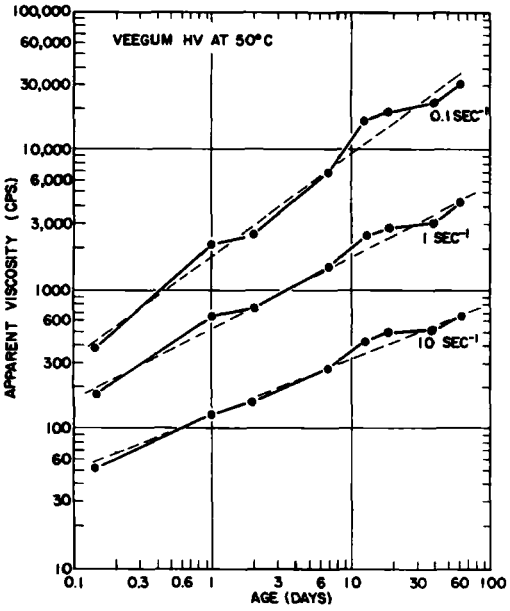

Fig. 4.—Variation of apparent viscosity at different shear rates with time for 2% Veegum HV suspensions prepared at 50°.

of samples prepared at the same time, a series of almost parallel lines for different temperatures of preparation and storage was obtained. An example is shown in Fig. 5. Arbitrarily the apparent viscosity may be compared for any desired shear rate. The general utility of this plot means that

$$\log \eta_D = a \log t + b \qquad \text{(Eq. 1)}$$

or

$$\eta_D = ct^a \qquad \text{(Eq. 2)}$$

where η_D is the apparent viscosity at shear rate D as measured at time t, a is the slope of the line from the plot, and b or c are constants which are dependent on the shear of making, temperature, and sample age.

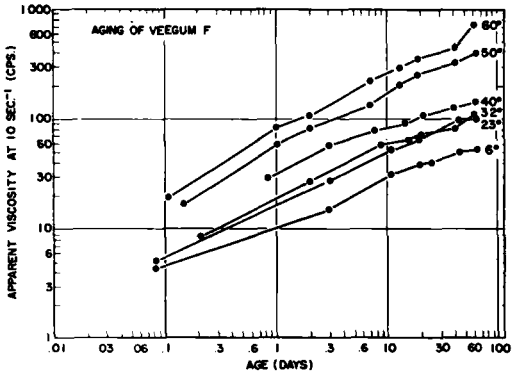

Fig. 5.—Variation of apparent viscosity at 10 sec.$^{-1}$ with time for 2% Veegum F suspensions prepared and held at various temperatures.

We have found that this equation is general for age dependent systems (9) and has been applicable over the time range of fractions of an hour to almost 2 years.

In Table I are given values of the constant a for both Veegums when viscosity is in centipoises and time in days.

It is felt that the values of a are temperature independent over the range examined, and in all probability the values are identical for the two Veegums. Because of the narrow range of a values it is impossible to tell the nature of the dependency on the logarithm of shear rate, since both plots of a directly and of log a are linear with log D over the range of data.

It is of interest to realize that the shear dependency of a does imply that a static yield value builds with time at a faster rate than does the dynamic viscosity.

Although every effort was made to duplicate making procedures, it was found that, in general, samples made over a year and a half apart tended not to duplicate but rather to yield their own set of parallel lines. This is probably due to minor variations in technique of initial hydration. This, however, did not affect the degree of temperature dependence reported in the next section.

Since the constants b or c are so history dependent, they are not reported here. Since the literature (3, 4) so emphasizes the critical dependence of mucilage viscosity upon the making procedure, it is evident that each method of preparation will generate its own set of b or c constants.

Samples stored at temperatures at 50° or higher tended to show slight syneresis after prolonged storage although they continued to yield viscosity determinations reasonably in line with the projection from earlier points.

Temperature Dependence.—Initially only four temperatures of preparation and storage were used: room temperature, 40, 73, and 88°. Somewhat later, when it was noted that, for any age, the log of the viscosities might obey an inverse temperature law, further samples were prepared. The samples prepared at different times yielded parallel lines for all plots, regardless of the age of the samples. Figure 6 shows for Veegum HV the dependence of the logarithm of the viscosity (at 10 sec.$^{-1}$) on the reciprocal of the absolute temperature for agings of

TABLE I.—DEPENDENCE OF a OF EQUATION 1 ON SHEAR RATE OF MEASUREMENT AND VEEGUM TYPE USED

Temp. of Measurement, ° C.	Veegum HV			Veegum F		
	0.1 sec.$^{-1}$	1 sec.$^{-1}$	10 sec.$^{-1}$	0.1 sec.$^{-1}$	1 sec.$^{-1}$	10 sec.$^{-1}$
6	0.74	0.58	0.48	0.85	0.60	0.43
23	0.76	0.68	0.43	0.76	0.56	0.45
32	0.58	0.48	0.32	0.77	0.58	0.38
40	0.61	0.38	0.30	0.58	0.48	0.36
50	0.62	0.40	0.32	0.79	0.58	0.45
60	0.83	0.62	0.36	0.70	0.63	0.53
Average	0.69	0.52	0.37	0.74	0.57	0.43

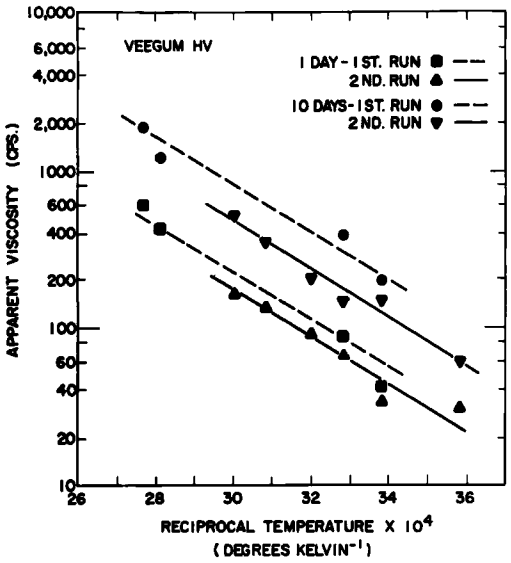

Fig. 6.—Variation of apparent viscosity at 10 sec.$^{-1}$ with reciprocal of absolute temperature for 2% Veegum HV suspensions for two different periods of aging.

both 1 and 10 days. The best fits are lines of similar slope through all points of a given set. The same slope was found for Veegum F.

Thus it is apparent that Veegum viscosity build-up follows an Arrhenius activation law relationship

$$2.303 \log \eta = -\frac{E}{RT} + d \qquad \text{(Eq. 3)}$$

and

$$\eta = fe^{-E/RT} \qquad \text{(Eq. 4)}$$

where η is in appropriate units, T is the absolute temperature, e is the base of natural logarithms, d and f are fit constants which are dependent on the units used for η and obviously upon the whole sample history, E is the apparent activation energy, and R the gas constant. It is apparent that torque at any shear rate, or similar units of gel measurement can be used for η and would affect only the fit constants d and f.

E is then found to have the magnitude of 8000 calories/mole. This would be the activation energy for process of opening up the crystal lattice to water. Since Veegum is slowly hydratable without heat, this is a plausible value for activation.

The normal temperature dependency of viscosity has been the subject of considerable work especially in the polymer field (10). Typical is the simple Andrade equation (11) which has a positive exponential since all polymer variants normally decrease in viscosity with increasing temperature. In contrast, this system shows a viscous build-up with temperature, the viscosity being a measure of the degree of hydration that has occurred.

Intrinsic Viscosity.—All samples in the range of concentration used showed slight thixotropic structure which was lost during the course of the full cycle of measurement. However, within the range of experimental determination, no significant difference between the heated and unheated samples could be detected at the low concentrations. This implies that in the very dilute stage all lattice structures are apparently swollen equally, within the week, or carry equivalent hydration shells, regardless of the temperature of storage during hydration.

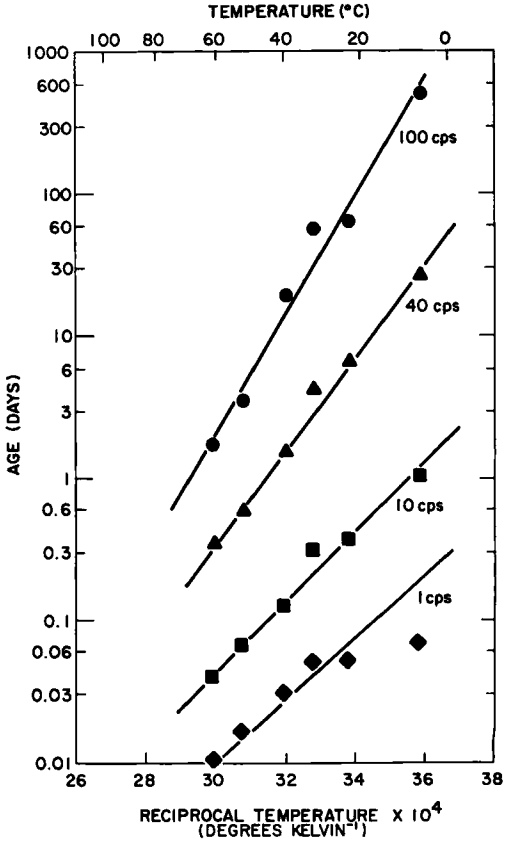

Fig. 7.—Variation of the time required to attain a given viscosity at 10 sec.$^{-1}$ as a function of the reciprocal of absolute temperature for 2% Veegum F suspensions.

General.—We may combine the equations for the viscosity dependence on aging at constant temperature and on temperature at equal ages into the form

$$2.303 \log \eta_D = 2.303\, a \log t - E/RT + g \quad \text{(Eq. 5)}$$

or

$$\eta_D = ht^a e^{-E/RT} \quad \text{(Eq. 6)}$$

where g and h are fit constants which are dependent upon units used and upon preparative history of the sample.

Equation 5 implies that, for any given viscosity level attained during aging, a plot of the reciprocal of temperature and the logarithm of the time required to attain that viscosity will be a straight line. In Fig. 7 are shown such plots for four viscosity levels of Veegum F. The lines are reasonably parallel as is required by the equation. Thus the times required to attain a given level of viscous build-up by varying temperature may be compared.

Equation 5 or 6 or their precursors imply an infinite viscosity with infinite time. This seems to have no practical likelihood of substantiation. Syneresis appears to be the phenomenon which dictates the boundaries within which Eqs. 5 and 6 are valid.

SUMMARY

The exponential aging relation has been found to hold for 2% Veegum HV and 2% Veegum F suspensions in water, so that the logarithm of the viscosity is a linear function of the logarithm of time over a time interval from a few hours to several months.

For samples of equivalent age, an Arrhenius-type relation holds to relate the viscosity with the temperature of preparation and storage. The apparent activation energy for both Veegum HV and Veegum F is about 8000 calories/mole.

The temperature and time relationship may be combined to one general equation which permits time to reach a given viscosity for a given temperature to be determined.

One constant of any of the above relationships is critically dependent on the method of mucilage preparation.

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Benzoic Acid as an Absorbance Standard in Infrared Spectrophotometry in Pharmaceutical Analyses

By J. P. COMER and A. M. RIBLEY

A solution of benzoic acid in chloroform measured at 5.91 μ was used to establish absorbance ratios for several drug substances. The ratios were used with daily measurements of benzoic acid as absorbance standards. The precision and accuracy of the technique was found to be equal to the customary method of using the drug substance as a reference standard.

THE PREDICTION of Carol (1) that the use of infrared spectrophotometry in pharmaceutical analyses would increase has certainly been correct. The maintenance of the multitude of chemical standards for infrared spectrophotometry has created many problems. The pharmaceutical laboratories must store and maintain in the original state these standard compounds and also supply governmental agencies with the proper standards. Many analysts (2–6) have reported on various methods for the standardization of instruments. Most of the work concerning the standardization of wavelength and possible use of solid standards does not consider some of the variables encountered in routine control of pharmaceuticals. Variable cell paths, temperature changes, and instrumental responses are corrected normally by performing standard readings of the known compound in solution. The purpose of this investigation was to attempt to find a suitable standard that could be used for quantitative analyses to replace the ever increasing number of individual standards that are encountered in pharmaceutical research programs.

Benzoic acid being readily available in high purity was selected as a trial substance since it has

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General.—We may combine the equations for the viscosity dependence on aging at constant temperature and on temperature at equal ages into the form

$$2.303 \log \eta_D = 2.303\, a \log t - E/RT + g \quad \text{(Eq. 5)}$$

or

$$\eta_D = ht^a e^{-E/RT} \quad \text{(Eq. 6)}$$

where g and h are fit constants which are dependent upon units used and upon preparative history of the sample.

Equation 5 implies that, for any given viscosity level attained during aging, a plot of the reciprocal of temperature and the logarithm of the time required to attain that viscosity will be a straight line. In Fig. 7 are shown such plots for four viscosity levels of Veegum F. The lines are reasonably parallel as is required by the equation. Thus the times required to attain a given level of viscous build-up by varying temperature may be compared.

Equation 5 or 6 or their precursors imply an infinite viscosity with infinite time. This seems to have no practical likelihood of substantiation. Syneresis appears to be the phenomenon which dictates the boundaries within which Eqs. 5 and 6 are valid.

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The exponential aging relation has been found to hold for 2% Veegum HV and 2% Veegum F suspensions in water, so that the logarithm of the viscosity is a linear function of the logarithm of time over a time interval from a few hours to several months.

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THE PREDICTION of Carol (1) that the use of infrared spectrophotometry in pharmaceutical analyses would increase has certainly been correct. The maintenance of the multitude of chemical standards for infrared spectrophotometry has created many problems. The pharmaceutical laboratories must store and maintain in the original state these standard compounds and also supply governmental agencies with the proper standards. Many analysts (2–6) have reported on various methods for the standardization of instruments. Most of the work concerning the standardization of wavelength and possible use of solid standards does not consider some of the variables encountered in routine control of pharmaceuticals. Variable cell paths, temperature changes, and instrumental responses are corrected normally by performing standard readings of the known compound in solution. The purpose of this investigation was to attempt to find a suitable standard that could be used for quantitative analyses to replace the ever increasing number of individual standards that are encountered in pharmaceutical research programs.

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distinct absorption maxima scattered through the 3–10 μ range. The carboxylic acid absorption at 5.91 μ was the most desirable reference wavelength since it represents the carbonyl region characteristic of so many drug substances and because it exhibits maximum sensitivity for benzoic acid. The results of the initial work with benzoic acid was so encouraging that studies of other potential standards were not undertaken.

EXPERIMENTAL

The Beckman IR 2 instrument was used for all the measurements unless noted otherwise. The benzoic acid solutions were prepared fresh daily to avoid concentration by loss of chloroform. A solution of *d*-propoxyphene hydrogen chloride (130 mg.) was made alkaline and extracted with chloroform. The chloroform was evaporated and the residue dissolved in 4 ml. of chloroform. Another solution was prepared containing acetylsalicylic acid (908 mg.), caffeine (640 mg.), and acetophenetidin (130 mg.) in 50 ml. of chloroform. Benzoic acid solutions were prepared containing 100 mg. (BA) and 600 mg. (BAII) in 10 ml. chloroform. The absorbances of these solutions were measured and ratios calculated. Accurate location of the absorption maxima was required for each determination made through the study.

Sufficient materials were obtained so that the same lot numbers could be used for the preparation of the solutions for the complete wavelengths studies. The solutions were prepared immediately before use.

TABLE I.—ABSORBANCES OF BENZOIC ACID IN CHLOROFORM WITH CELL PATH 0.1 MM.

Concn. mg./ml.	Microns	Slit, mm.	Absorbance
10	5.91	0.3	0.393
10	5.91	0.35	0.366
10	5.91	0.4	0.347
10	5.91	0.45	0.327
10	5.91	0.50	0.311
25	6.91	0.45	0.153
25	7.08	0.45	0.311
25	7.59	0.45	0.306
60	3.92	0.15	0.405
60	6.25	0.3	0.293
60	8.50	0.7	0.312
60	8.87	0.6	0.242
60	9.34	0.8	0.180
60	9.75	0.8	0.171

RESULTS AND DISCUSSION

The use of a single standard would not be practical unless the absorption ratios of compound to standard would be constant in respect to time and changes of absorption cells thickness. Wichers (7) reported the use of benzoic acid as a thermometric standard but indicated possible instability problems. It was found that chloroform solutions of benzoic acid were stable. (Zero time A = 0.394, 1 hr. A = 0.392, 5 hr. A = 0.393, and 48 hr. A = 0.396.) The maxima of benzoic acid in chloroform solutions observed on a Beckman IR 2 instrument are shown in Table I.

The *d*-propoxyphene hydrogen chloride and the acetylsalicylic acid, caffeine, and acetophenetidin solutions were prepared at an interval of 30 months and the ratios of absorbances were calculated. Table II lists the comparative results. The changes in the ratios were not considered significant for the number of observations so a study was started for the selection of the optimum standard wavelengths to be used for a series of compounds. Table III shows the concentration, wavelength, cell path, etc. The calculated ratios from the absorb-

TABLE III.—CHLOROFORM SOLUTIONS USED IN ABSORBANCE STUDIES

Compound	Concn./ml.	Microns	Slit
Acetylsalicylic acid (ASA)	20 mg.	5.70	0.3
d-Propoxyphene HCl	32.5 mg.	5.80	0.3
Benzoic acid (BAI)	10 mg.	5.91	0.3
Salicylic acid	8 mg.	6.02	0.3
Caffeine	4 mg.	6.04	0.4
Benzoic acid (BAII)	60 mg.	6.34	0.3
Acetophenetidin (API)	10 mg.	6.61	0.6
Acetophenetidin (APII)	12.8 mg.	6.61	0.6
Benzoic acid (BAIII)	60 mg.	8.51	0.6
Aniline (AN)	0.08 ml.	8.52	0.6
Benzoic acid (BAIV)	60 mg.	9.75	0.8
Phenaglycodol (PHEI)	25 mg.	9.88	0.9
Isopropanol (ISO)	0.02 ml.	10.60	0.9
Phenaglycodol (PHEII)	100 mg.	10.93	0.9
Ethanol (ETH)	0.04 ml.	11.40	1.5

TABLE II.—EFFECT OF TIME ON RATIOS OF ABSORBANCES OF BENZOIC ACID TO OTHER COMPOUNDS

Compound[a]	Months Elapsed	Microns	Slit, mm.	Denominator	Ratio of Absorb.
d-Propoxyphene	0	5.80	0.3	BA	0.814
d-Propoxyphene	30	5.81	0.3	BA	0.813
Acetylsalicylic acid	0	5.69	0.3	BA	1.06
Acetylsalicylic acid	30	5.71	0.3	BA	1.07
Caffeine	0	6.04	0.4	BA	1.10
Caffeine	30	6.05	0.4	BA	1.14
Acetophenetidin	0	6.61	0.6	BAII	1.19
Acetophenetidin	30	6.61	0.6	BAII	1.18
Benzoic acid (BA)		5.91	0.4		
Benzoic acid (BAII)		6.34	0.35		

[a] See text for preparation of solutions.

TABLE IV.—RATIOS OF ABSORBANCES USING BECKMAN IR 2 INSTRUMENT

Time Elapsed, Weeks	API[a] / BAII	API / BAI	AN / BAIII	AN / BAI	PHEI / BAIV
0	0.8540	0.7015	...	...	1.431
1	0.8602	0.7013	...	...	...
2	0.8825	0.7020	0.7034	0.6288	1.415
3	0.8870	0.7069	0.7168	0.6375	1.415
4	0.8782	0.6990	0.7135	0.6352	1.412
5	0.8836	0.7096	0.7252	0.6464	1.424
Av.	0.874	0.703	0.714	0.637	1.419
S.D./Av. × 100	1.57	0.569	1.26	1.14	0.5562

Time Elapsed, Weeks	PHEI / BAI	ETH / BAIV	ETH / BAI	ISO / BAIV	ISO / BAI
0	0.6352	...	...	1.810	0.8036
1	...	...	...	1.751	0.8025
2	0.6288	1.631	0.7247	1.807	0.8030
3	0.6401	1.631	0.7378	1.801	0.8342
4	0.6378	1.638	0.7398	1.819	0.8214
5	0.6364	1.638	0.7323	1.780	0.7954
Av.	0.634	1.63	0.734	1.79	0.810
S.D./Av. × 100	0.662	0.248	0.919	1.39	1.81

[a] See Table III for identification of solutions.

TABLE V.—PRECISION OF RATIO METHOD

Time Elapsed, Weeks	ASA Soln. 1 / BAI	ASA Soln. 2 / BAI	ASA Soln. 1 / ASA Soln. 2
0	1.016	1.009	1.007
2	1.005	1.016	0.989
4	1.016	1.021	0.995
6	1.010	1.019	0.991
8	1.014	1.016	0.998
12	1.007	1.007	0.999
Av.	1.011	1.014	0.9966
S.D.	0.0047	0.0055	0.0064

ance data are shown in Table IV. Precision is expressed as coefficient of variation (100 × S.D./Av.) since the ratios differ in magnitude.

General observations indicated that a single benzoic acid solution (BAI) at 0.3 slit and 5.91 μ could be used as a suitable standard. Precision and accuracy studies were started based on these observations.

The precisions of ratios derived from absorbance of acetylsalicylic acid and benzoic acid (BAI) were obtained over a three-month span. The ratios in Table V were calculated from the average absorbance of three solutions. There is no statistically significant difference (F-test) in the precision of the ratios calculated by using benzoic acid as the reference or by using acetylsalicylic acid as reference.

Another cell having 80% of the cell path of the one used for data in Table V was used for obtaining absorbances and ratios for four averages of three solutions each. The ratios found for ASA/BA were 0.995, 1.007, 1.006, 1.012, illustrating that cell path changes occurring in practical use will not significantly change the ratios.

After the ratios were established by numerous replicate determinations between BAI and each of the several compounds previously mentioned, a measure of the accuracy of this system for its proposed use was made. Observed absorbance values at weekly intervals for the individual standard materials were compared to absorbance values calculated by multiplying the current BAI absorbance by the previously established ratios.

Observation of the data from this experiment shown in Table VI indicates an accuracy and precision equivalent to that expected in customary standardization techniques.

Since the manually operated Beckman IR 2 was used for the preceding experiments, the precision of the technique was evaluated on the Perkin-Elmer model 21 recording instrument. Ratios of the five drugs *vs.* BAI were determined on three different days using an average of three replicates for benzoic acid and a single observation of the samples. An extensive realignment of the instrument was made between the first and second series. The data in Table VII show that satisfactory precision may be obtained using benzoic acid as a standard on a recording instrument if careful control of instrument parameters (recording speed, pen sensitivity, slit width, etc.) is maintained.

SUMMARY

Chloroform solutions of benzoic acid (BAI) were shown to be stable over two-day periods, but were prepared fresh daily to avoid concentration by evaporation. Ratios of *d*-propoxyphene, caffeine, and acetophenetidin solutions were found to be constant over a 30-month period (Table II). The precision of ratios calculated from maxima of benzoic acid nearest the maxima of various drug substances were compared to those ratios calculated from benzoic acid at 5.91 μ and the maxima of the drug substances (Table IV). Statistical analysis showed a significant difference for acetophenetidin and ethanol but not for aniline, phenaglycodol, or isopropanol. For acetophenetidin the best precision was by the ratio calculated from the 5.91 μ BA solution.

TABLE VI.—ACCURACY STUDY

Solution[a]	Absorbance Observed	Absorbance Calcd.	Absorbance Diff.	% Error
d-Propoxyphene HCl	0.285	0.280	−0.005	−1.8
d-Propoxyphene HCl	0.280	0.279	−0.001	−0.4
d-Propoxyphene HCl	0.277	0.279	+0.002	+0.7
d-Propoxyphene HCl	0.283	0.280	−0.003	−1.1
Acetylsalicylic acid	0.378	0.381	+0.003	+0.8
Acetylsalicylic acid	0.376	0.380	+0.004	+1.1
Acetylsalicylic acid	0.375	0.380	+0.005	+1.3
Acetylsalicylic acid	0.389	0.388	−0.001	−0.3
Salicylic acid	0.251	0.252	+0.001	+0.4
Salicylic acid	0.250	0.251	+0.001	+0.4
Salicylic acid	0.248	0.251	+0.003	+1.2
Phenaglycodol (PHEII)	0.488	0.480	−0.008	−1.6
Phenaglycodol (PHEII)	0.478	0.480	+0.002	+0.4
Phenaglycodol (PHEII)	0.474	0.479	+0.005	+1.1
Caffeine	0.277	0.277	0.000	0.0
Caffeine	0.267	0.267	−0.000	0.0
Caffeine	0.277	0.276	−0.001	−0.4
Caffeine	0.275	0.276	+0.001	+0.4
Acetophenetidin (APII)	0.319	0.323	+0.004	+1.3
Acetophenetidin (APII)	0.320	0.321	+0.001	+0.3
Acetophenetidin (APII)	0.323	0.322	−0.001	−0.3

[a] See Table III for identification of solutions.

TABLE VII.—PRECISION OF RATIOS WITH A RECORDING INSTRUMENT

Time Elapsed, Months	Salicylic Acid[a] BAI	Caffeine[a] BAI	Acetophenetidin[a] II BAI	Acetylsalicylic[a] Acid BAI	Phenaglycodol[a] BAI
0	0.609	0.849	1.21	0.895	1.16
1	0.621	0.962	1.19	0.920	1.16
2	0.622	0.848	1.21	0.904	1.18
$\frac{\text{S.D.}}{\text{Av.}} \times 100$	1.17	0.916	0.962	1.40	0.987

[a] Ratio of absorbance.

The difference for ethanol is not considered practically significant for the coefficient of variation for the ethanol/BAI is only 0.92%. It was concluded from this study that the absorbance of BAI at 5.91 μ could be used for a reference standard for all drugs listed in this experiment.

The ratios for acetylsalicylic acid/BAI and acetylsalicylic acid/acetylsalicylic acid were measured during a three-month period (Table V). No statistically significant difference (F-test) was observed, and the coefficient of variation was less than 1%. Cell path changes occurring in practical use did not change the ratios.

Accuracy studies (Table VI) showed an average difference between customary standardization techniques and the ratio method of +0.16% with a pooled standard deviation of ±0.9%.

Ratios must be established for each instrument, for changes in slit width, optics, or measuring systems of various instruments will change the ratios. For example the ratio found for acetylsalicylic acid/BAI for single beam Beckman IR 2 was 1.01 and the ratio found for double beam Perkin-Elmer No. 21 was 0.907.

Noticeable changes in ratios on a single instrument can be used as an indication of needed maintenance or instrumental calibration by techniques described in references (2–6).

CONCLUSION

The accuracy and precision of the use of benzoic acid solutions in chloroform as secondary reference standards for quantitative infrared spectrophotometry was found to be for practical purposes equivalent to the use of individual standards of the drugs being measured. The successful use of the technique of using the secondary reference depends upon the establishment of accurate ratios on a single instrument by suitable replication and careful control of slit width and instrument calibration.

The technique would be useful to laboratories doing large numbers of measurements on a variety of drug substances that require the preparation of individual standards.

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Spectrophotometric Determination of Sodium 2,6-Dimethoxyphenyl Penicillin Monohydrate (Methicillin)

By W. J. WEAVER and R. F. RESCHKE

Sodium 2,6-dimethoxyphenyl penicillin in pH 3.8 acetate buffer shows no absorbance in the region 290 mμ to 360 mμ. When heated in this buffer an absorption maximum appears at 330 mμ. The increase in absorption is directly proportional to the penicillin concentration. This procedure has been used to determine potencies in finished products. Results are compared with the iodimetric and microbiological methods.

HERRIOTT (1) investigated the degradation of salts of penicillin G, X, K, and F under controlled acidic conditions. Procedures are given for the determination of high and low potency material, in which solutions are diluted in 0.4 *M* acetic acid-sodium acetate buffer, pH 4.6, and heated for 15 minutes at 100°. The difference in absorbance at 322 mμ between heated and unheated buffered solutions provides a means of measuring total penicillin present. The results are within 5.0% of theoretical values. Herriott noted in his work that the change in absorbance did not increase linearly with concentration.

Stock (2) modified the method proposed by Herriott. His work demonstrates the importance of the presence of trace amounts of copper in the penicillin-penicillenic acid reaction. When copper is added to the buffer solution, reproducible results are obtained in the determination of benzylpenicillins in penicillin oral tablets. Stock demonstrated the effects of variable conditions such as time of immersion in water bath, volume of penicillin buffer mixture, and concentration of copper. By adding a small amount of copper, not only is the reproducibility improved, but the sensitivity is increased approximately ten per cent.

More recently Holbrook (3) investigated the experimental conditions and expanded the original method to include determination of various salts of benzylpenicillin in ointments, lozenges, oily injections, and suspensions. Penicillin potencies obtained by the spectrophotometric method are compared with those obtained by microbiological methods.

We have studied the application of these methods to one of the new synthetic penicillins, methicillin,[1] for the purpose of developing a routine analytical procedure. The main advantages of the proposed method are simplicity, speed, and sensitivity. The procedure has been successfully employed for analyzing large numbers of samples.

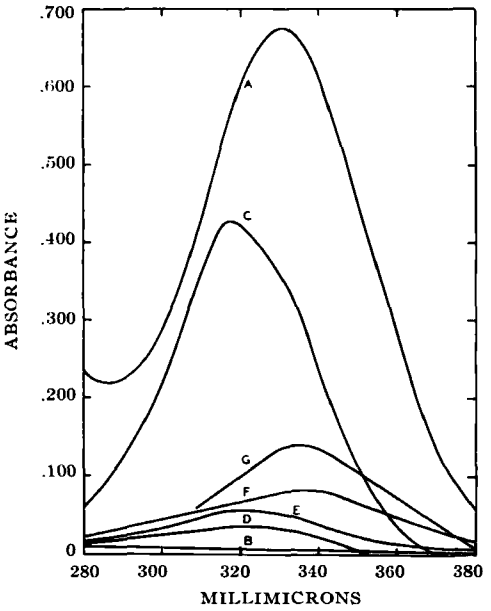

Fig. 1—Absorption spectra of penicillins: 4.76×10^{-5} moles per ml., 0.2 *M* buffer, pH 3.8, 30 min., 70°C. A, methicillin; B, 6-aminopenicillanic acid; C, benzylpenicillin potassium; D, potassium phenoxyethylpenicillin; E, potassium phenoxyethylpenicillin; F, D(-)α-aminobenzylpenicillin (free acid); and G, potassium methylphenylisoxazolylpenicillin.

EXPERIMENTAL

Reagents.—*Dilute Copper Sulfate Solution:* Dissolve 0.392 Gm. of copper sulfate pentahydrate in 100 ml. of distilled water. This solution contains 1 mg. of copper per ml.

Buffer Solution, pH 3.8: Mix 13 ml. of 2 *M* sodium acetate and 87 ml. of 2 *M* acetic acid. Add 0.5 ml. of the dilute copper sulfate solution and dilute to 1 L. with distilled water. The resulting solution is 0.2 *M* in respect to acetate and

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[1] Marketed as Staphcillin by Bristol-Myers Co.

contains 0.5 mcg. of copper per ml. Adjust the pH within the range 3.7–3.9 with sodium acetate or acetic acid, as required.

Preparation of Standard Curve.—Prepare an aqueous solution containing 500 mcg. of standard methicillin per ml. Transfer a 2.0-ml. aliquot to each of two 100-ml. volumetric flasks. Dilute the contents of one flask to volume with the buffer solution. Dilute the other to volume with distilled water. Transfer 10 ml. of the buffer dilution to a 16 mm. × 150 mm. test tube and place in a 70° water bath for 30 minutes. Remove and cool immediately in an ice bath to quench the reaction. Determine the absorbance within 1 hour in a 1-cm. cell at 330 mμ, using the water dilution as reference. Establish additional points on the calibration curve by using 3.0- and 4.0-ml. aliquots of the methicillin standard solution. Plot absorbance *versus* mcg. per ml.

Procedure.—Prepare an aqueous solution containing approximately 500 mcg. methicillin per ml. Dilute 3.0 ml. of the solution to 100 ml. with the buffer solution. Prepare a similar 3.0 to 100 dilution with water, which serves as the reference solution. Proceed as directed in preparation of the standard curve, and determine the concentration from the prepared graph.

RESULTS AND DISCUSSION

Figure 1-A illustrates the change that occurs when methicillin is heated under optimum conditions of pH, time, and temperature. Equivalent amounts of other natural and synthetic penicillins heated under these conditions produce absorption spectra shown in Fig. 1, B-G. Note that methicillin develops an absorption maximum at 330 mμ, whereas maxima of the other penicillins appear at different wavelengths in the 310–340 mμ range. Table I contains the absorptivities at the absorption maximum for each penicillin.

TABLE I.—ABSORPTIVITIES AND ABSORPTION MAXIMA OF VARIOUS PENICILLINS IN 0.2 *M* ACETATE BUFFER, pH 3.8, AFTER HEATING FOR 30 MIN. AT 70°C.

	Wavelengths at Absorption Maximum in 310–340 mμ Range	Absorptivities at λ Max.
2,6-Dimethoxyphenylpenicillin, sodium salt	330 mμ	330
6-Aminopenicillanic acid	None	...
Benzylpenicillin, potassium salt	317 mμ	240
Phenoxymethylpenicillin, potassium salt	320 mμ	11
Phenoxyethylpenicillin, potassium salt	320 mμ	21
D(−) α-Aminobenzylpenicillin (free acid)	335 mμ	44
Methylphenylisoxazolylpenicillin, potassium salt	334 mμ	64

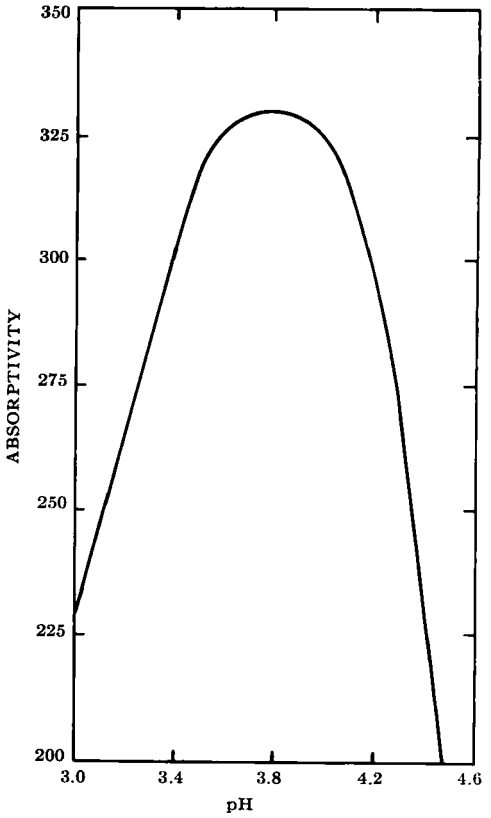

Fig. 2.—Effect of pH: 0.2 *M* buffer, 30 min., 70°C.

The conditions necessary to produce maximum sensitivity and reproducibility for methicillin were found to be 30 minutes heating at 70° in 0.2 *M* acetate buffer, pH 3.8, containing 0.5 mcg. copper

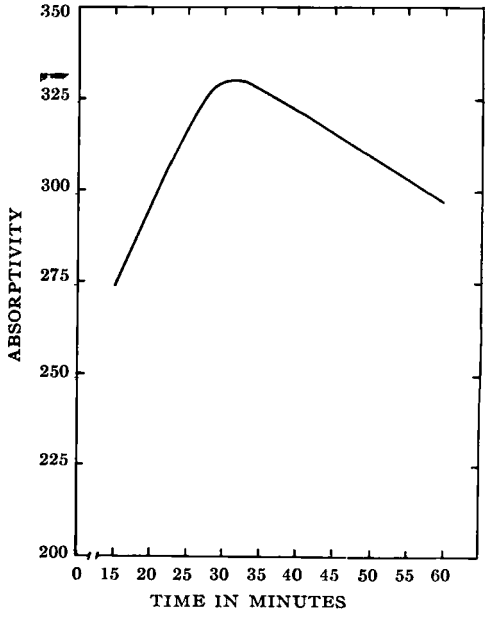

Fig. 3.—Effect of heating time: 0.2 *M* buffer pH 3.8, 70°C.

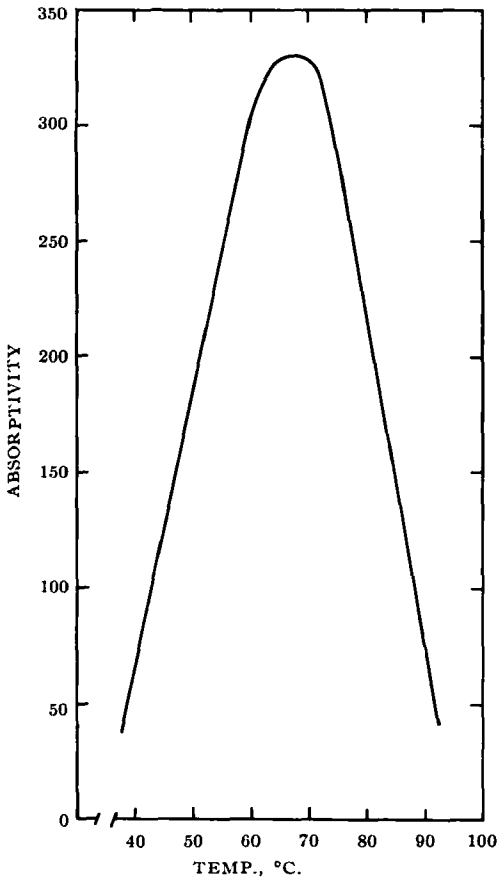

Fig. 4.—Effect of temperature: 0.2 *M* buffer, pH 3.8, 30 min.

per ml. The effect of acetate buffers over a pH range of 3.0 to 4.6 was studied, the results being shown in Fig. 2. Figure 3 shows the effect of time of heating at 70° in 0.2 *M* acetate buffer, pH 3.8. The maximum absorptivity develops after 30 minutes. Between 28 and 40 minutes the range of variation in the absorptivity is approximately 1.3%. Figure 4 indicates the effect on the absorptivity at 330 mμ when methicillin is heated in pH 3.8 buffer at various temperatures for 30 minutes. Heating at 25°, 50°, and 100° at other pH levels produces lower absorptivities.

The effect on absorptivity using buffers ranging from 0.1 to 1.0 molar is illustrated in Fig. 5.

The method was also applied to buffered methicillin containing 5% sodium citrate. The results are compared with iodimetric and biological assays

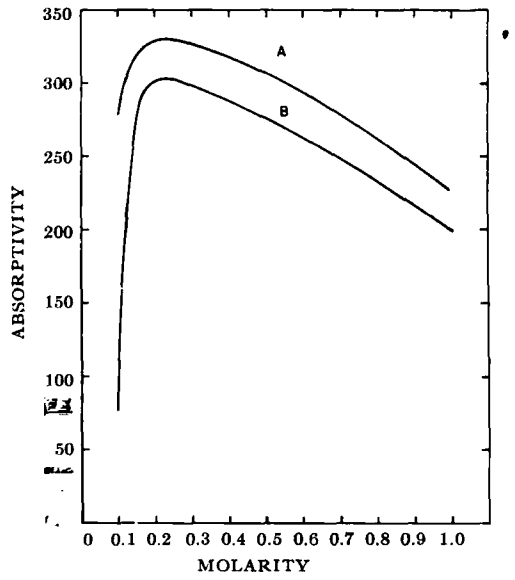

Fig. 5.—Effect of buffer molarity: pH 3.8, 30 min., 70°C. *A*, 0.5 mcg. copper per ml. added; *B*, without copper.

in Table II. It can be seen that sodium citrate causes no interference.

TABLE II.—COMPARISON OF THE RESULTS BY THE SPECTROPHOTOMETRIC METHOD WITH THOSE BY IODIMETRIC AND BIOASSAY

Sample	Spectrophotometric, mcg./mg.	Iodimetric, mcg./mg.	Bioassay, mcg./mg.
Buffered methicillin	850	854	850
Buffered methicillin	834	830	860
Buffered methicillin	847	837	830
Unbuffered methicillin[a]	877	880	870

[a] Theoretical potency of methicillin (sodium salt, monohydrate) is 905 mcg./mg.

Precision.—A statistical study of the recommended procedure was made by assaying eight samples ranging from 10 to 20 mcg. methicillin per ml. The apparent relative standard deviation(s) calculated as percentage of average absorptivity is ±1.8%.

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Identification and Differentiation of Some Phenothiazine-Type Tranquilizers

By D. K. YUNG and M. PERNAROWSKI

Methods are described for the identification and differentiation of the 13 phenothiazine-type tranquilizers listed in Schedule F of the Canadian Food and Drugs Act. These tranquilizers may be identified by observing the colors formed with certain reagents and by determining the melting points and infrared spectral characteristics of either the picrate or reineckate derivatives.

Most of the substituted phenothiazines possess tranquilizing properties and, consequently, are subject to controls in the market place. The pharmaceutical analyst, particularly if he is employed by an enforcing agency, is often called on to identify substances such as those listed herein. The methods available to the analyst depend mostly on the formation of a characteristic color with a selected reagent.

Feigl (1) described two spot tests for the base material, phenothiazine. Deviller (2) developed methods for the identification of thirteen substituted phenothiazines. Filho and Aichinger (3) showed that phenothiazine, promethazine, diethazine, isothiazine, chlorpromazine, and neozine could be identified by reacting these compounds with a series of reagents. Identification procedures for chlorpromazine hydrochloride, based on color formation, are described in the "British Pharmacopoeia 1958" (4) and the "United States Pharmacopeia XVI" (5). Because of the similarity in chemical structure, many substituted phenothiazines have been included in studies on the identification of antihistamines by color formation (6, 7). Rajeswaran and Kirk (8, 9) reported the identification of 50 tranquilizing and related drugs by means of color reactions and microscopic crystal tests.

Little systematic work has been carried out on the identification of substituted phenothiazines by derivative formation. The preparation of picrate and reineckate derivatives of some of the substituted phenothiazines has been reported (10, 11). Some picrates (12) and reineckates (11) were prepared and used in quantitative procedures involving the colorimetric determination of selected substituted phenothiazines.

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A number of procedures are described in this paper that will aid the analyst in the rapid identification of a restricted group of phenothiazines possessing tranquilizing properties.

EXPERIMENTAL

The following substituted phenothiazines were investigated: acepromazine hydrochloride, chlorpromazine hydrochloride, fluphenazine, levomepromazine maleate, mepazine acetate, perphenazine, prochlorperazine dimaleate, promazine hydrochloride, thiopropazate dihydrochloride, thioridazine hydrochloride, trifluoperazine dihydrochloride, triflupromazine hydrochloride, and trimeprazine tartrate. A Thomas-Hoover capillary melting point apparatus was used to determine the melting points of these compounds. The values so obtained are shown in Table I and are in good agreement with those supplied by the manufacturers or reported in the "Merck Index" (13). The chemical names of these substances may be found in the latter reference.

Reagents for Identification by Color Formation.—(*a*) Reagent I: Add 10 drops of 10% w/w hydrochloric acid to 30 ml. of a 1% aqueous ferric chloride solution; (*b*) Reagent II: 1% aqueous cobalt acetate solution; 10% isopropylamine in acetone; (*c*) Reagent III: 10% aqueous chloramine-T solution; (*d*) Reagent IV: add 1 ml. of hydrochloric acid U.S.P. to 50 ml. of a 0.1% aqueous palladium chloride solution; (*e*) Reagent V: nitric acid; (*f*) Reagent VI: add three ml. of concentrated sulfuric acid to 17 ml. of a 6% solution of uranium nitrate in 95% ethanol. This reagent will not form the desired colors unless it is freshly prepared. (*g*) Reagent VII: 0.5% w/w ammonium vanadate in concentrated sulfuric acid; (*h*) Reagent VIII: Add five drops of 0.1 *N* sulfuric acid to 20 ml. of a 4% aqueous silver nitrate solution.

Identification by Color Formation.—Prepare a 0.15% aqueous solution of the substituted phenothiazine. Heat gently, if necessary, to completely solubilize the phenothiazine. Transfer five drops of this solution to a depression in a white spot plate and add one drop of reagent. Observe the color immediately after mixing. (For the purposes of this paper, one drop is equal to approximately 0.05 ml.)

For reactions involving Reagent II, add one drop of the cobalt acetate solution and then one drop of the 10% isopropylamine in acetone to five drops of the test solution.

For reactions involving silver nitrate, transfer ten drops of the phenothiazine solution to a semimicro

TABLE I.—MELTING POINTS OF THE SALTS, PICRATES, AND REINECKATES OF SOME SUBSTITUTED PHENOTHIAZINES

	M.p., °C. (uncor.)		
	Salt[a]	Picrate	Reineckate
Acepromazine	135.0–136.0	69.0–73.0	154.0–156.0[b]
Chlorpromazine	196.0–196.5[b]	175.5–176.0	207.0–210.5[b]
Fluphenazine	231.0–232.0[b]	234.0–235.0[b]	179.0–181.0[b]
Levomepromazine	188.0–188.5[b]	69.0–79.0[b]	164.5–168.0[b]
Mepazine	70.5–71.5	145.0–146.0[b]	153.0–155.5[b]
Perphenazine	95.5–96.5	239.0[b]	154.5–156.5[b]
Prochlorperazine	219.0–221.0[b]	245.0[b]	156.0–159.5[b]
Promazine	178.5–180.0	145.5–146.0	166.5–168.0[b]
Thiopropazate	222.0–224.0[b]	223.0[b]	149.0–150.5[b]
Thioridazine	162.0–163.0	85.0–95.0	174.5–175.0[b]
Trifluoperazine	169.0–171.0	242.0[b]	183.5–186.0[b]
Triflupromazine	173.0–174.0	152.0–153.0	194.5–195.0[b]
Trimeprazine	162.0–163.0	130.0	168.5–170.0[b]

[a] See first paragraph under *Experimental*. [b] Melt with decomposition.

test tube and add two drops of the reagent. Heat the solution in boiling water for 5 minutes and observe the color.

The colors formed with seven of the eight reagents are reported in Table II. The results obtained for Reagent I are shown in Fig. 1.

Identification by Picrate Formation.—Dissolve 500 mg. of the substituted phenothiazine in 50 ml. of distilled water. If the substance is not completely soluble, stir for 10 minutes and filter. Add slowly, with stirring, 50 ml. of a saturated solution of picric acid in water. Allow the mixture to stand for ten minutes, filter using a sintered-glass filter of medium porosity, and wash the precipitate with a small quantity of distilled water. Recrystallize twice from 95% ethanol and dry the crystals in a desiccator over phosphorous pentoxide.

If recrystallization is not possible, wash the recovered substance thoroughly with distilled water, then with 95% ethanol, and dry as indicated above.

The melting points of the 13 picrate derivatives are listed in Table I.

Purity Criterion for Picrate Derivatives.—Carbon, hydrogen, and nitrogen determinations were carried out on the 13 picrate derivatives. Experimental values agreed satisfactorily with the calculated theoretical values.

The equivalent weights of the picrate derivatives were determined in the following way: (*a*) Method for monopicrates: Dissolve 30 mg., accurately weighed, in 15 ml. of glacial acetic acid and 15 ml. of chloroform. Titrate the solution with 0.02 *N* acetous perchloric acid using four drops of a 0.5% solution of crystal violet in glacial acetic acid as indicator. The color change, from purple to blue, was checked potentiometrically using a Precision-Shell dual titrometer equipped with glass and sleeve-type calomel electrodes. Carry out a blank determination and make any necessary corrections in the calculation. (*b*) Method for dipicrates: Dissolve 50 mg., accurately weighed, in 30 ml. of dimethylformamide. Titrate the solution with 0.1 *N* potassium hydroxide in methanol using four drops of a freshly prepared 0.5% solution of thymol blue in methanol as indicator. The color change, from greenish yellow to green, was checked potentiometrically as indicated above. Protect the contents of the flask from atmospheric conditions. Carry out a blank determination and make any necessary corrections in the calculation.

The equivalent weight is equal to the weight of the sample in grams divided by the product of the volume and normality of the titrant. The equivalent weight of each picrate was determined by using the appropriate method and the experimental and theoretical values compared. All results were found to be satisfactory.

Identification by Reineckate Formation.—Dissolve 150 mg. of the substituted phenothiazine in 50 ml. of 0.1 *N* hydrochloric acid. If the substance is not completely soluble, stir for 10 minutes, and then filter. Add slowly, with stirring, 50 ml. of a saturated solution of ammonium reineckate in water. Refrigerate for 10 minutes, filter through

TABLE II.—COLOR REACTIONS[a] OF SOME SUBSTITUTED PHENOTHIAZINES

	Reagent II	Reagent III	Reagent IV	Reagent V	Reagent VI	Reagent VII	Reagent VIII
Acepromazine	GN.	L.Y.	BR.	O.BR.	Y.O.	BR.	N.R.
Chlorpromazine	L.BL.	GR.	V.	R.	R.	R.	BE.CL.
Fluphenazine	L.BL.	L.Y.	O.	O.BR.	L.O.	Y.BR.	CR.W.
Levomepromazine	L.V.	L.Y.	V.	V.	V.	V.	V.
Meprazine	L.BL.	CR.P.	BL.	O.BR.	O.BR.	BR.	L.PI.
Perphenazine	L.BL.	N.R.	L.O.	PI.	N.R.	PI.	N.R.
Prochlorperazine	L.BL.	L.Y.	O.BR.	R.	R.	R.	P.PI.
Promazine	L.BL.	CR.P.	D.BL.	BR.	O.BR.	BR.	PI.
Thiopropazate	L.BL.	L.Y.	O.BR.	R.	P.R.	R.	BE.CL.
Thioridazine	L.BL.	CR.BL.	R.BR.	BL.	BL.	BL.	BL.CL.
Trifluoperazine	L.BL.	CR.BE.	O.BR.	O.BR.	O.	Y.BR.	CR.W.
Triflupromazine	L.BL.	CR.V.	O.BR.	O.BR.	O.	Y.BR.	CR.W.
Trimeprazine	BL.	L.GR.	BL.	O.BR.	L.PI.	BR.	P.PI.

[a] BE. = beige; BL. = blue; BR. = brown; GN. = green; GR. = gray; O. = orange; P. = purple; PI. = pink; R. = red; V. = violet; W. = white; Y. = yellow; CL. = cloudy; CR. = creamy; D. = dark; L. = light; N.R. = no reaction.

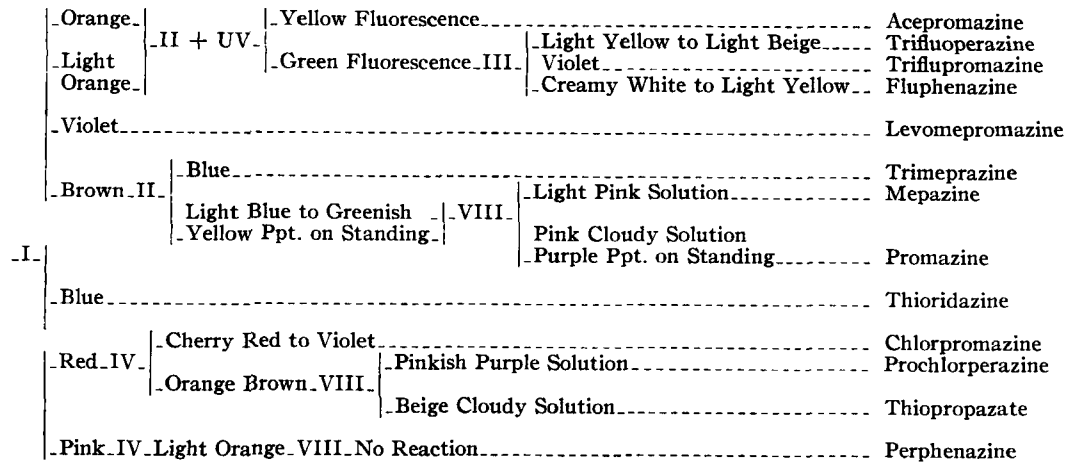

Fig. 1.—Sequence of color tests for differentiation of some substituted phenothiazines. Roman numerals refer to reagents mentioned in text. UV: sample exposed to ultraviolet radiant energy using a Mineralight, model SL, black light lamp.

a sintered-glass filter of medium porosity, wash the precipitate with distilled water, and recrystallize twice from 70% ethanol at 55°. Dry the crystals in a desiccator over phosphorous pentoxide.

The decomposition points of the 13 reineckate derivatives are listed in Table I.

Purity Criterion for Reineckate Derivatives.—The chromium content of each of the reineckates was determined by ashing the sample at 800° for 3 hours. The per cent chromium in the derivatives (calculated from the weight of ash) compared favorably with theoretical values.

Procedure for the Preparation of Infrared Spectrum.—Mix 15 mg. of the picrate or reineckate with 285 mg. of previously dried A.C S. grade potassium bromide in a Wig-L-Bug amalgamator. Transfer the sample to a Beckman rectangular pellet die, evacuate, and subject the mixture for 10 minutes to a pressure of 20,000 lb./sq. in. using a Carver laboratory press. Record the spectrum of the pellet on a Beckman IR 5 infrared spectrophotometer.

The infrared spectra of promazine picrate and trifluoperazine reineckate are shown in Fig. 2. Portions of other spectra are illustrated in Figs. 3 and 4.

DISCUSSION

One of the easiest ways to identify an organic

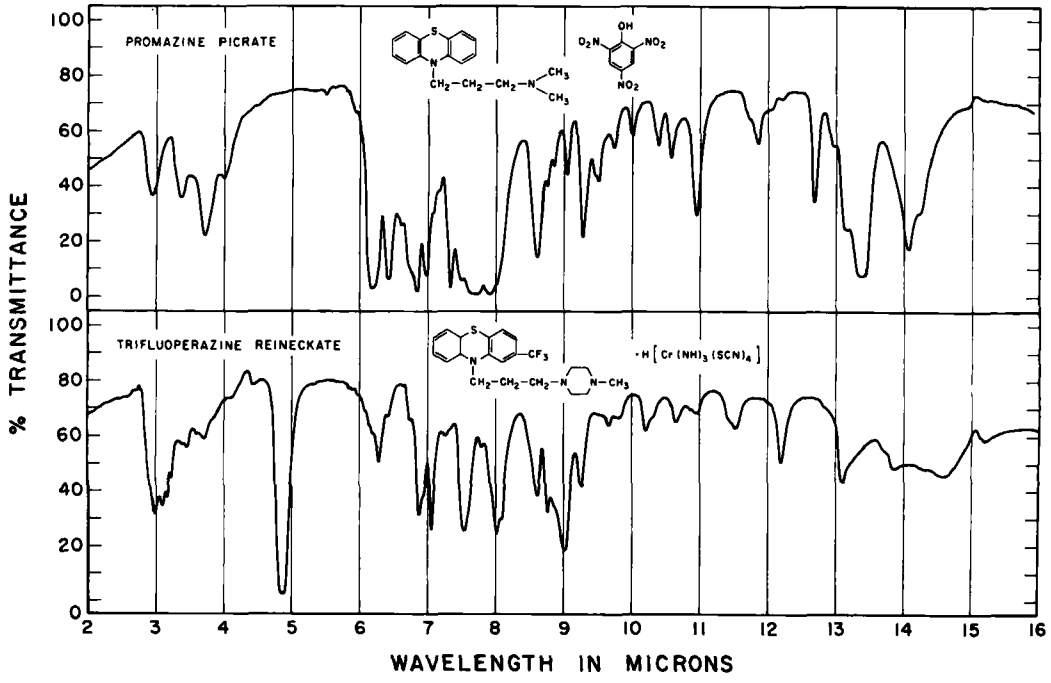

Fig. 2.—Infrared spectra of promazine picrate and trifluoperazine reineckate.

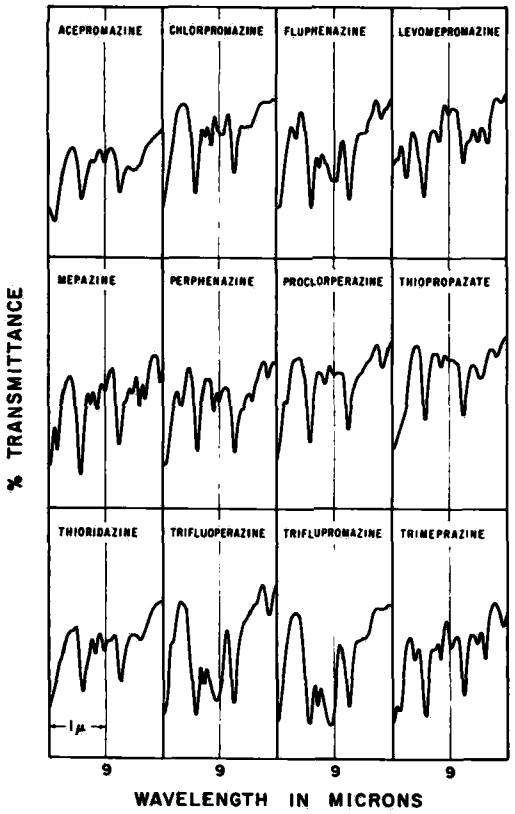

Fig. 3.—Infrared spectra of the picrates of some substituted phenothiazines.

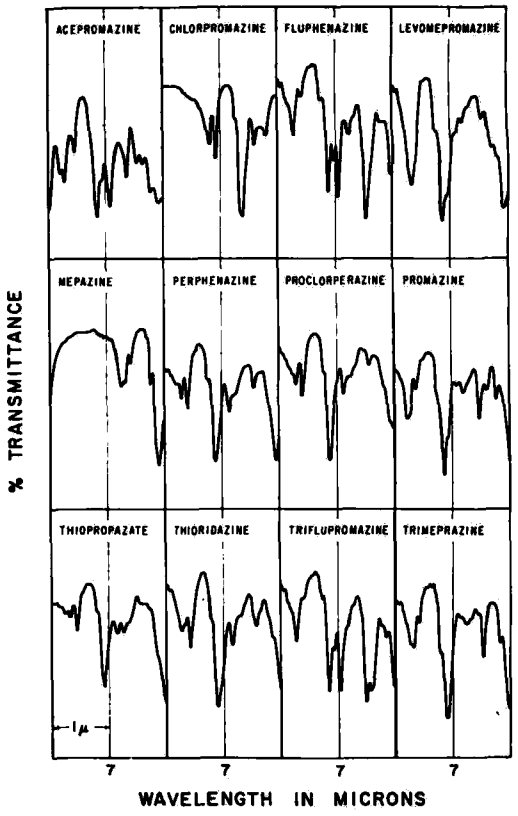

Fig. 4.—Infrared spectra of the reineckates of some substituted phenothiazines.

compound is to carry out a reaction which yields a characteristically colored product. The rationale in most of these tests is that the particular reaction is specific but, unfortunately, such specificity is limited to the conditions outlined by the researcher. Moreover, reaction to color is largely a matter of opinion and, consequently, what is red to one chemist might be orange to another. Tests in sequence are thus better since there is less chance of error in interpretation.

Two sets of data are presented herein. The first set (See Fig. 1) outlines a sequence of color tests that are useful in the identification and differentiation of the substituted phenothiazines listed in this paper. Only some of the reagents listed in the previous section are required for this testing sequence. The second set (See Table II) is a tabulation of the colors formed with seven of the eight reagents and may be useful to the control chemist in setting up a different sequence of tests to cover particular problems involving a more restricted group of phenothiazines.

Tests based on color formation depend upon the amount of material available and on the degree of dilution. In order to standardize conditions, tests were carried out on solutions containing 0.15% of active ingredient. This concentration value was derived from the solubility of the least soluble phenothiazine, prochlorperazine dimaleate. On this basis, reactions were carried out on solutions containing approximately 0.5 to 1.0 mg. of sample. Some of the reagents will detect much smaller quantities of material but some change in color will occur and, consequently, control samples should be run at the same time. A total of approximately 15 mg. of ingredient is generally sufficient to carry out the color tests indicated in Fig. 1.

Levomepromazine gave a violet color with all reagents except chloramine-T. This latter reagent did, however, produce an initial light violet color which then turned to the light yellow color indicated in Table II. Similarly, thioridazine gave a blue color with all reagents except palladium chloride. These two substituted phenothiazines can, therefore, be quickly and fairly accurately characterized on the basis of these particular color tests.

Fluorescent solutions resulted when the substituted phenothiazines were reacted with Reagent II and then exposed to ultraviolet radiant energy. Solutions containing acepromazine yield a bright yellow fluorescence and, on this basis, can be readily differentiated from the other 12 phenothiazines. This technique is of no further value in the identification of any single phenothiazine. It does, however, divide the remaining 12 phenothiazines into two groups, the first yielding a green fluorescence with fluphenazine, trifluoperazine, and triflupromazine and the second giving a basically blue color with the remaining substances. These observations are useful in the differentiation scheme outlined in Fig. 1. It was also observed that aqueous solutions of these substituted phenothiazines fluoresced when exposed to ultraviolet

radiant energy but this did not appear to be useful for identification purposes because the colors formed were not characteristic. Prolonged exposure to ultraviolet radiant energy will result in colored solutions but, again, this does not particularly aid the analyst in the identification of these substances. This color formation is probably due to oxidation reactions caused by the radiant energy.

Color reactions cannot be considered as final criterion in the identification of drugs and, for this reason, picrate and reineckate derivatives of the substituted phenothiazines were prepared. As expected, the monobasic phenothiazines, that is, acepromazine, chlorpromazine, levomepromazine, mepazine, promazine, thioridazine, triflupromazine, and trimeprazine formed monopicrates. The dibasic phenothiazines formed dipicrates. These derivatives can usually be purified by recrystallization from 95% ethanol. Difficulty was experienced, however, in the purification of acepromazine, levomepromazine, perphenazine, prochlorperazine, and thioridazine picrates. These derivatives were washed with water, and then with 95% ethanol, to remove undesired impurities. Levomepromazine picrate is soluble in 95% ethanol and cannot be washed with this solvent. Elementary analyses and equivalent weight determinations indicated that such washings were sufficient for purification purposes.

Dipicrates are insoluble in the glacial acetic acid-chloroform solvent system used in the determination of equivalent weight values. It was, therefore, necessary to develop a secondary technique involving the use of dimethylformamide as solvent and potassium hydroxide in methanol as titrant. The latter technique involves a titration of the picric acid portion of the molecule whereas the acetous perchloric acid method implies a determination of the phenothiazine itself. Both methods gave satisfactory results.

All reineckate derivatives were prepared in acidic medium. It has been reported that high temperatures will decompose some reineckates (14, 15) and, consequently, recrystallizations were carried out at 55°. After two recrystallizations, all derivatives were obtained as the monoreineckates. Chromium determinations on the crude materials indicated that five of the dibasic substituted phenothiazines, namely fluphenazine, perphenazine, prochlorperazine, thiopropazate, and trifluoperazine, first formed direineckates. Trifluoperazine, a dibasic compound, initially formed a monoreineckate. Recrystallization converted the direineckates described above to monoreineckates. Such conversion was also observed by Lee (15) in his study on the use of ammonium reineckate in the identification of nitrogenous organic bases.

Although the melting points of these derivatives are useful in the identification of these substances, overlapping of values does occur and, consequently, creates difficulties but only if such values are viewed alone. The analyst may turn to color formation or to infrared spectroscopy in order to resolve these difficulties. In one case, at least, crystal color aids in differentiating one picrate from another. Mepazine forms a bright yellow picrate, whereas the promazine derivative has a rusty red color.

Infrared spectra are useful in further identifying these derivatives. For the most part, the spectra of these compounds are similar and, consequently, only those of promazine picrate and trifluoperazine reineckate are illustrated over the full 2–16 μ region. It was observed, however, that the spectra of the picrates and reineckates were sufficiently different in the 8–10 and 6–8 μ regions, respectively, to permit identification of the parent substance. Figures 3 and 4 illustrate the significant portions of these spectra.

Certain bands are common to all of the picrates of the substituted phenothiazines. Strong bands occur throughout the 3.00 (OH stretching), 3.30 to 4.00 (CH stretching), 5.90 to 6.26 (C—C stretching), 6.42 (nitro stretching), 6.62 to 7.00 (benzene ring frequency), 7.35 (nitro stretching), and 13.40 to 14.10 (benzene ring frequency) μ regions. It is probable that all of these bands arise from the picric acid portion of the molecule since they are all present in the spectrum of this acid alone. In spite of all of this, special characteristics in the 8–10 μ region makes identification possible. Thus, promazine picrate can be differentiated from fluphenazine picrate by the appearance of bands of weak and medium intensity at 8.85 and 9.50 μ, respectively. Similarly, trimeprazine picrate yields a spectrum which shows two weak bands at 8.10 and 8.40 μ, bands which are not present in the spectrum of promazine picrate.

The spectra of the reineckates of the substituted phenothiazines have certain bands in common. Strong bands occur in the 4.86 and 8.00 to 8.10 (nitrile stretching) μ regions. Broad, intense bands, due to an NH stretching vibration, may be observed in the 2.96 to 3.50 μ region. As in the case of the picrates, differentiation must be based on special features in a restricted portion of the spectra. Even though heavy atoms have a damping effect on the spectrum (16), there is sufficient detail in the 6–8 μ region to permit identification of these substances. For example, fluphenazine reineckate has a band of medium intensity of 6.96 μ whereas trifluoperazine reineckate shows no such absorption. It is difficult to distinguish between perphenazine and prochlorperazine reineckates on the basis of the features in this particular region. However, a weak band does occur at 8.11 μ in the spectrum of perphenazine reineckate. No problems should occur with the evaluation of the other spectra of the derivatives of these substituted phenothiazines.

Several of the substituted phenothiazines deserve special mention. Thus, fluphenazine, trifluoperazine, and trifluopromazine possess a $-CF_3$ group within their molecules. The presence of this particular functional group is indicated in the infrared spectrum by a band at approximately 7.50 μ. Similarly, the presence of the carbonyl groups of acepromazine and thiopropazate may be established on the basis of sharp, intense bands near 5.90 μ. All of these bands occur in the spectra of either the picrate or the reineckate derivatives.

The techniques developed herein were applied to 12 commercial preparations containing various substituted phenothiazines. One of these prepara tions contained fluphenazine in concentrations equal to 0.25 mg. per tablet. This preparation could not be identified by using the techniques described herein because of the difficulties encountered in extracting this quantity of active ingredient

from the tablet mass. No difficulties were encountered with the remainder of the preparations.

CONCLUSION

Several methods are described for the identification of 13 substituted phenothiazines with tranquilizing properties. The methods are rapid and the manipulative techniques are simple.

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Stability Assays of Pharmaceutical Preparations by Quantitative Paper Chromatography II

Quantitation by Spectrophotofluorometry

By HENRY R. ROBERTS and MARIA RITA SIINO

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The sensitivity of the method permits the chromatography and quantitation of 10 and 20 mcg. quantities. This is important since estrogens, because of their physiological potency, are generally compounded in low concentrations, for example, 0.5% in oil formulations and 0.05% in tablets.

Fluorometric methods may be classified according to whether the fluorescence measured is that of the compound in its native state or is induced by chemical transformations. Most accepted procedures for the quantitation of estrogens fall in this second category. The fluorescence is developed in sulfuric acid and measured in the visible range (2).

The development of the spectrophotofluorometer, capable of activating and measuring fluorescence throughout the visible and ultraviolet regions, has revealed the presence of useful ultraviolet fluorescence in many compounds not previously known to fluoresce in solution.

Duggan, *et al.* (3), in studying the fluorescent properties of a number of compounds of biologi-

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from the tablet mass. No difficulties were encountered with the remainder of the preparations.

CONCLUSION

Several methods are described for the identification of 13 substituted phenothiazines with tranquilizing properties. The methods are rapid and the manipulative techniques are simple.

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Stability Assays of Pharmaceutical Preparations by Quantitative Paper Chromatography II

Quantitation by Spectrophotofluorometry

By HENRY R. ROBERTS and MARIA RITA SIINO

A quantitative paper chromatographic method has been developed which is routinely used as a stability assay for estrogenic hormones in castor oil and tablet formulations. The method involves: (*a*) A separation of the estrogenic hormone from interfering degradation products. (*b*) The location of the estrogenic hormone on the chromatogram by the guide strip technique employing a chromogenic agent. (*c*) The elution of the estrogenic hormone from the chromatogram. (*d*) A quantitative spectrophotofluorometric analysis of the eluate. Recoveries in excess of 95% are obtained by simultaneously chromatographing replicates of standard and sample solutions on the same chromatogram. A detailed account of the procedure is presented using as examples the assay of estradiol valerate in castor oil formulations and ethinyl estradiol in tablets.

RECENTLY we have described methods for the quantitative determination of hormone preparations using paper chromatographic separations followed by spectrophotometric analysis (1). In this paper we wish to report on techniques which permit the quantitation of estrogens in ethanol eluates by spectrophotofluorometry. The examples chosen are the quantitative determination of two estradiol derivatives, estradiol valerate in castor oil and ethinyl estradiol in tablets.

The sensitivity of the method permits the chromatography and quantitation of 10 and 20 mcg. quantities. This is important since estrogens, because of their physiological potency, are generally compounded in low concentrations, for example, 0.5% in oil formulations and 0.05% in tablets.

Fluorometric methods may be classified according to whether the fluorescence measured is that of the compound in its native state or is induced by chemical transformations. Most accepted procedures for the quantitation of estrogens fall in this second category. The fluorescence is developed in sulfuric acid and measured in the visible range (2).

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TABLE I.—PAPER CHROMATOGRAPHIC CONDITIONS FOR ASSAY OF ESTROGENIC HORMONES

Compound	Paper Impregnating Solution	Developing Solvent	Time, hr.
Estradiol Valerate	20% Carbitol in Chloroform	Methylcyclohexane saturated with Carbitol	3
Ethinyl Estradiol	25% Propylene Glycol in Chloroform	Toluene saturated with Propylene Glycol	16

cal interest found that estradiol fluoresced in the ultraviolet region in the absence of sulfuric acid.

Based on this observation, a sensitive quantitative paper chromatographic assay has been developed which is used routinely for the analyses of estradiol derivatives in castor oil and tablet formulations.

EXPERIMENTAL

The paper chromatographic procedure involves essentially four steps: (*a*) The separation of the intact estrogenic hormone from interfering degradation products. (*b*) The location of the intact estrogenic hormone on the chromatogram by the guide strip technique employing a chromogenic agent. (*c*) The elution of the intact estrogenic hormone from the chromatogram. (*d*) A quantitative spectrophotofluorometric analysis of the eluate.

Preparation of Standard

The reference standard solution should contain 0.2 mg. of the estrogenic hormone per ml. of solution. In a volumetric flask, the appropriate amount of steroid is dissolved in the smallest volume of N,N-dimethylformamide. Then methyl isobutyl ketone is added to the mark. The reference standard material should be from the same lot as the formulation undergoing analysis.

Preparation of Sample

Oil Preparations.—For the assay of estradiol valerate in castor oil, the amount of sample which will yield, based on theory, 0.2 mg. of the hormone per ml. of solution is weighed into a volumetric flask, dissolved and diluted to the mark with methyl isobutyl ketone.

Tablet Preparations.—For the assay of ethinyl estradiol in tablets containing ethinyl estradiol and norethindrone acetate,[1] 10 tablets are reduced to a fine powder using a mortar and pestle and an amount of the powdered tablet mix which contains, based on theory, 250 mcg. of the steroid are transferred to a 60 ml. glass-stoppered bottle. Two ml. of N,N-dimethylformamide are added, the bottle stoppered, and shaken mechanically for 10 minutes, after which 3 ml. of methyl isobutyl ketone are added and the bottle shaken mechanically for an additional 10 minutes. The extract is then filtered under reduced pressure through a 15 ml. Buchner funnel (fine porosity fritted disk). If the filtrate is not clear, an asbestos pad should be used with the filter.

The filter paper used is the same as described previously (1). Methylcyclohexane saturated with Carbitol [2-(2-ethoxyethoxy) ethanol] (4) and toluene saturated with propylene glycol (5) are the two developing solvents used. Any conventional chamber used for descending paper chromatography is suitable. The chromogenic agent is made up of one part of Folin-Ciocalteu phenol reagent plus 4 parts of water (6). The eluting solvent used was 95% ethanol.

Paper Chromatographic Procedure.—Two chromatograms are run per sample. The slotted filter paper, which has been described previously (1), contains six 3/4 in. strips which are spotted for the estradiol valerate assay at 1-in. intervals along the line of origin as follows:

ORIGIN 1 2 3 4 5 6

No. 1 Chromatogram	No. 2 Chromatogram
1 = Sample, 0.05 ml.	Sample, 0.10 ml.
2 = Standard, 0.05 ml.	Standard, 0.05 ml.
3 = Standard, 0.10 ml.	Standard, 0.10 ml.
4 = Sample, 0.05 ml.	Sample, 0.10 ml.
5 = Sample, 0.05 ml.	Sample, 0.10 ml.
6 = Paper blank	Paper blank

For the ethinyl estradiol assay, both strips are spotted as follows:

ORIGIN 1 2 3 4 5 6

1 = Sample, 0.20 ml.; 2 = Standard, 0.05 ml.; 3 = Standard, 0.10 ml.; 4 = Sample, 0.20 ml.; 5 = Sample, 0.20 ml.; 6 = Paper blank.

Blow-out pipets, of 1- and 2-ml. capacity, graduated in 0.01 ml., are used to apply the standards and the sample to the paper. The spotted strips are impregnated with the stationary phase by dipping them into the appropriate chloroform solution (Table I). The procedure has been described in detail previously (1). Following solvent development (Table I), the strips are removed from the chamber and dried in a mechanical convection oven for 20 minutes at 90°.

The positions of the estrogens on the chromatogram are located by employing the guide strip technique. The strip corresponding to the No. 1 spot on each chromatogram is cut out, dipped into the diluted phenol reagent, and then exposed to

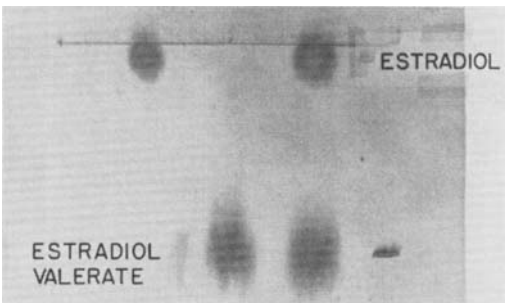

Fig. 1.—Chromatogram showing the separation achieved between estradiol valerate and estradiol in the solvent system methylcyclohexane saturated with Carbitol. Folin-Ciocalteau phenol reagent was used as the color reagent.

[1] Marketed as Gestest by E. R. Squibb & Sons.

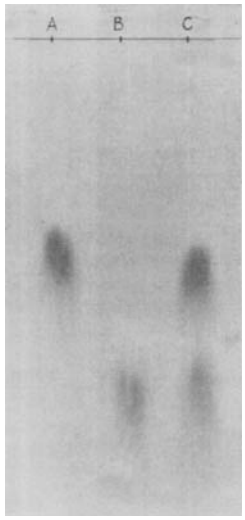

Fig. 2.—Chromatogram showing the separation achieved between ethinyl estradiol and estradiol in the solvent system toluene saturated with propylene glycol. Folin-Ciocalteau phenol reagent was used as the color reagent. A = estradiol, B = ethinyl estradiol, and C = mixture of the two.

ammonia vapors for 5 minutes by placing them into a chamber containing concentrated ammonium hydroxide. Estrogens appear on the chromatogram as blue spots against a light gray background (Figs. 1 and 2). This color development procedure is a slight modification of the procedure for the detection of estrogens on chromatograms described by Mitchell (6).

The color developed guide strip is air dried and then realigned with the untreated portion of the chromatogram. The positions of the estrogens are marked off with a solder pencil, cut out and folded, and placed in 50 ml. Erlenmeyer flasks. A paper blank, equal in area to the standard and sample segments, is included for each chromatogram. Ten milliliters of 95% ethanol are added to each flask and the estrogens are eluted off the filter paper segment by shaking on a reciprocating shaker for 30 minutes (1).

The amount of estrogens present in the eluates is determined by spectrophotofluorometry. An Aminco-Bowman spectrophotofluorometer (Cat. No. 4-8100) is used under the following conditions: clear silica cells, 10 mm. light path; 1P28 photomultiplier tube; No. 3 slit arrangement; and activation at 280 mμ and measurement of the resulting fluorescence at 310 mμ for estradiol valerate and 305 mμ for ethinyl estradiol.

The instrument is adjusted to a scale reading (on the photomultiplier-microphotometer) of 90–95, using the maximum standard eluate. The intensity of fluorescence is measured on an arbitrary 0–100 scale, using the photometer.

Calculation of Estradiol Valerate in Castor Oil Formulations.—The photometer readings are recorded on a data sheet which contains all the information necessary to calculate the concentration of estradiol valerate in the sample. Table II gives the data obtained in typical analyses.

For each of the two chromatograms, readings are obtained for the standard at two levels and for the sample at one level in duplicate. The readings of the two standards for each of the chromatograms are converted to the equivalent reading for the 0.1 ml. standard concentration and averaged. The two photometer readings of the sample are converted to 0.1 ml. readings and averaged.

The concentration of estradiol valerate in the sample is calculated from the following equation

$$\text{mg. estradiol valerate per ml. formulation} = \frac{A \times B \times C \times D}{E \times F \times G}$$

where: A = average photometer reading of 10 ml. eluate of 0.1 ml. chromatographed sample; B = weight in mg. of chromotographed 0.1 ml. standard; C = volume in ml. of sample dilution; D = specific gravity of formulation; E = average photometer reading of 10 ml. eluate of 0.1 ml. chromatographed standard; F = weight in Gm. of sample; and G = volume (0.1 ml.) of chromatographed sample.

The final concentration of estradiol valerate in the sample is obtained by averaging the values obtained for each of the two chromatograms.

Sample Calculation.—In Table II, chromatogram No. 1:

Table II.—Data Sheet for Estradiol Valerate Assay[a]

Chrom.	Flask No.	Cell No.	Spot	Photometer Reading: Uncorrected	Corrected For Blank	Converted St. = 0.10 ml. Sa. = 0.10 ml.	Average St. = 20 γ Sa. = 0.1 ml.	Conc.[b] mg./ml. Formulation
1	1	1	St. = 0.05 ml.	70	30	60	57.75	40.5
1	2	2	St. = 0.10 ml.	95.5	55.5	55.5		
1	3	3	Sa. = 0.05 ml.	66	26	52	54	
1	4	4	Sa. = 0.05 ml.	68	28	56		
1	5	5	Paper Blank	40	...			
2	6	1	St. = 0.05 ml.	63	28	56	56.75	40.3
2	7	2	St. = 0.10 ml.	92.5	57.5	57.5		
2	8	3	Sa. = 0.10 ml.	89	54	54	53	
2	9	4	Sa. = 0.10 ml.	87	52	52		
2	10	5	Paper Blank	35	..	..		

[a] Project Hv. 45–53; sample Es. 31–35/15A–1 initial; assay, estradiol valerate; sample weight, 1.1900 Gm./250 ml.; sp. gr. sa., 1.0295; weight standard, 20.0 mg./100 ml.; filter paper, ethanol-washed finger strips impregnated with Carbitol (20% in chloroform); developing solvent, methylcyclohexane saturated with Carbitol; developing time, 3 hours; drying condition, 20 minutes at 90°C; eluting solution, 10 ml. of 95% EtOH; eluting time, 30 minutes. Spectrophotofluorometer specifications: Aminco-Bowman instrument, Cat. No. 4-8100; 10 mm. silica cells; 1P28 tube; No. 3 slit; activate, 280 mμ; fluorescence, 310 mμ. [b] Average: 40.4.

TABLE III.—DATA SHEET FOR ETHINYL ESTRADIOL ASSAY[a]

Chrom.	Flask No.	Cell No.	Spot	Photometer Reading: Uncorrected	Corrected for blank	Converted St. = 0.05 ml. Sa. = 0.20 ml.	Average St. = 10.1 γ Sa. = 0.20 ml.	Conc.[b] mg./Tablet
1	1	1	St. = 0.05 ml.	41	33	33	32.5	0.052
1	2	2	St. = 0.10 ml.	72	64	32		
1	3	3	Sa. = 0.20 ml.	41	33	33	33	
1	4	4	Sa. = 0.20 ml.	41	33	33		
1	5	5	Paper Blank	8	..	...		
2	6	1	St. = 0.05 ml.	41	34	34	32.75	0.050
2	7	2	St. = 0.10 ml.	70	63	31.5		
2	8	3	Sa. = 0.20 ml.	40	33	33	32.5	
2	9	4	Sa. = 0.20 ml.	39	32	32		
2	10	5	Paper Blank	7	..	...		

[a] Project Es.402a-11; sample Es.402a-11-C-1, 4 months at 22°C.; assay, ethinyl estradiol; sample weight, 499.5 mg./5 ml.; theoretical tablet weight, 100 mg.; weight standard, 20.3 mg./100 ml.; filter paper, ethanol-washed finger strips impregnated with propylene glycol (25% in chloroform); developing solvent, toluene saturated with propylene glycol; developing time, 16 hours; drying condition, 20 minutes at 90°C.; eluting solution, 10 ml. of 95% EtOH; eluting time, 30 min. Spectrophotofluorometer specifications: Aminco-Bowman instrument, Cat. No. 4-8100; 10 mm. silica cells; 1P28 tube: No. 3 slit; activate, 280 mμ; fluorescence, 305 mμ. [b] Average: 0.051.

mg. estradiol valerate per ml. =

$$\frac{54.0 \times 0.020 \times 250 \times 1.0295}{57.75 \times 1.190 \times 0.1} = 40.5$$

Calculation of Ethinyl Estradiol in Gestest Tablets.—For each of the two chromatograms, photometer readings are obtained for the standard at two levels and for the sample at one level in duplicate. The readings of the two standards for each of the chromatograms are converted to the equivalent reading for the 0.05 ml. (or 10 γ) standard concentration and averaged. The two readings of the sample are also averaged.

The concentration of ethinyl estradiol per tablet is calculated from the following equation

$$\text{mg. ethinyl estradiol/tablet} = \frac{A \times B \times C \times D}{E \times F \times G}$$

where: A = average photometer reading of 10 ml. eluate of 0.2 ml. chromatographed sample extract; B = weight in mg. of chromatographed 0.05 ml. standard; C = theoretical weight in mg. of 1 tablet; D = volume in ml. of sample extract; E = average photometer reading of 10 ml. eluate of 0.05 ml. chromatographed standard; F = weight in mg. of sample; and G = volume (0.2 ml.) of chromatographed sample extract.

The final concentration of ethinyl estradiol per tablet is obtained by averaging the values obtained for each of the two chromatograms.

Sample Calculation.—In Table III, chromatogram No. 1

mg. ethinyl estradiol per tablet =

$$\frac{33 \times 0.0101 \times 100 \times 5}{32.5 \times 499.5 \times 0.2} = 0.052$$

The reported value, 0.051 mg. per tablet (see Table III), is the average of the values obtained for each of the chromatograms.

RESULTS AND DISCUSSION

In general, the fluorescent peak-height, as measured on the microphotometer, will be a linear function of concentration. This is true for the range of concentrations employed in the assays described here. This was demonstrated by preparing two chromatograms, each containing 10, 20, 30, 40, and 50 mcg. of ethinyl estradiol, and carrying them through the complete assay procedure. The two corrected readings were averaged for each concentration and plotted against the concentration, yielding the resulting straight line, as seen in Fig. 3.

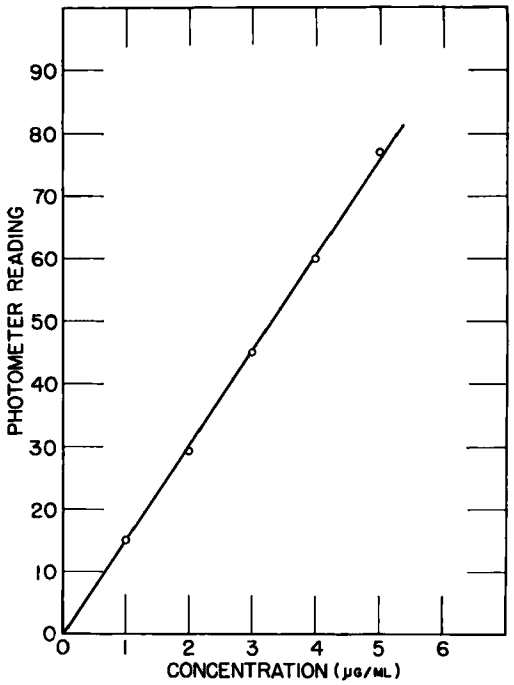

Fig. 3.—Variation of fluorescence with concentration. Ninety-five per cent ethanol eluates of chromatographed ethinyl estradiol.

The activation and fluorescence wavelengths

TABLE IV.—PAPER CHROMATOGRAPHIC ANALYSIS OF KNOWN ESTRADIOL VALERATE SOLUTIONS

Theoretical, mg.	Chromatogram, mg. found 1	2	Average, mg.	Dev. from Theory, %
25.2	24.6	24.7	24.65	−2.2
22.5	22.5	23.0	22.75	+1.1

TABLE V.—PAPER CHROMATOGRAPHIC ANALYSES OF ESTRADIOL VALERATE IN CASTOR OIL UNDERGOING STABILITY STUDIES

Sample No.	Theoretical Conc., mg./ml.	Time, Months	Temperature, °C. 5	22	40	50
			Found, mg./ml.			
1	5	6	5.1	5.3	5.3	5.0
2	5	12	...	4.8	4.8	5.0
3	5	6	...	5.0	5.2	5.1
4	16	6	...	16.9	16.5	15.8
5	20	8	...	20.2	20.5	20.8
6	20	6	20.9	20.7	20.6	20.4
7	20	6	20.7	21.0	20.0	20.4
8	40	6	...	40.6	39.3	38.6
9	40	6	41.7	41.3	38.1	40.0
10	40	12	...	40.1	39.9	40.4

(280–310 mμ for estradiol valerate and 280–305 mμ for ethinyl estradiol) are only apparent wavelengths. No corrections (7) have been made to provide true activation and fluorescence spectra. The apparent activation and fluorescence maxima may, therefore, vary slightly from instrument to instrument and should be determined by each investigator for his instrument.

The sensitivity of fluorescent measurement results in accurate analysis at eluate concentration ranges of 1 and 2 mcg. per ml. This, in turn, eliminates the need of any elaborate extraction procedure and permits the dilution of the oil formulation and the subsequent chromatography and resolution of estradiol valerate without interference from the oil base (which occurs at higher applications of diluted formulations to the filter paper).

While estradiol valerate in castor oil formulations is chromatographed at 10 and 20 mcg. levels, the sample extracts of ethinyl estradiol are chromatographed only at 10 mcg. levels. Ethinyl estradiol is present in Gestest at a concentration of 50 mcg. per tablet. Five hundred milligrams of powdered tablet (equivalent to 5 tablets) extracted with 5 ml. of solvent yields 50 mcg. of ethinyl estradiol per ml. of extract. Therefore, in order to chromatograph 10 mcg. of steroid, 200 μl. are spotted. To spot 20 mcg. would require 400 μl. of solution to be spotted. For this reason, the sample extract is spotted in duplicate at the 10 mcg. level.

As discussed previously (1), recoveries in excess of 95% must be obtained if any analytical method is to serve as an effective stability assay. The assay procedure described here yields results having an error less than ±5% and can accurately differentiate between preparations differing by 10%.

This was demonstrated by preparing two known estradiol valerate solutions, one at 25.2 mg./ml. and the other, 10% less, at 22.5 mg./ml. The analysis of the first solution, Table IV, gave a result of 24.65 mg. of estradiol valerate, a deviation of 2.2% from the theoretical concentration of 25.2 mg. The second solution assayed 22.75 mg. or 1.1% deviation from the theoretical concentration of 22.5 mg.

Accuracies of this magnitude are obtained since the standards and sample are run on the same chromatogram in replicate and both are exposed simultaneously to the same chromatographic and assay conditions. Table V contains additional data indicating that assays with recoveries in excess of 95% are obtained with consistency. The data also show that estradiol valerate in castor oil formulations at various concentrations is extremely stable and no breakdowns have been observed upon prolonged storage at elevated temperatures.

The stability of ethinyl estradiol in Gestest tablets plus the sensitivity and accuracy of the technique is illustrated in the data presented in Table VI. The reliability of the assay is assured when 50 mcg. of ethinyl estradiol can be measured with an accuracy of ±5% or ±2.5 mcg.

TABLE VI.—PAPER CHROMATOGRAPHIC ANALYSES OF ETHINYL ESTRADIOL IN TABLETS UNDERGOING STABILITY STUDIES

Sample No.	Initial Assay (mg./Tablet)	Time, Months	Temperature, °C. 22	40
			mg./Tablet	
1	0.049	4	0.051	0.051
2	0.049	4	0.050	0.050
3	0.051	5	0.048	0.049
4	0.049	6	0.048	0.050
5	0.049	12	0.050	0.049
6	0.049	12	0.051	0.048

Not only does the procedure provide for an accurate analysis of ethinyl estradiol in tablets, but it achieves this without the need of any elaborate extraction. The extraction with N,N-dimethylformamide followed by dilution with methyl isobutyl ketone is all the sample preparation required.

The spectrophotofluorometric procedure for the assay of estrogenic hormones has not been applicable to those preparations which contain sesame oil. Some component or components present in the oil interfere in the spectrophotofluorometric measurement, resulting in assay figures greatly in excess of theory.

SUMMARY

1. A quantitative paper chromatographic spectrophotofluorometric procedure has been developed for the assay of estrogenic hormones.

2. The procedure is used routinely to measure the stability of a number of estrogenic hormones in castor oil and tablet formulations. The assay of estradiol valerate in castor oil and ethinyl estradiol in tablets are described in detail.

3. No extraction of the estrogenic hormones

in castor oil is required. A simple dilution with methyl isobutyl ketone is the only sample preparation required.

4. Estrogenic hormones in tablets are extracted simply with N,N-dimethylformamide followed by dilution with methyl isobutyl ketone and filtration.

5. Accuracies in excess of 95% are obtained by simultaneously chromatographing replicates of standard and sample solutions on the same chromatogram.

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Technical Articles

Adaptation of Commercial Viscometers for Special Applications in Pharmaceutical Rheology II

Severs Extrusion Rheometer

By JOHN H. WOOD, GREGORY CATACALOS, and S. V. LIEBERMAN†

The Severs rheometer has been slightly modified to provide greater flexibility of use. Besides its normal use as a rheometer, it may be used to accept samples in collapsible tubes. Such samples undergo no change in thixotropic set before actual measurement. The apparatus can also be used to shear samples of up to 700-ml. volume in a manner comparable to a high-speed filler. The method of calibrating the filler shears is outlined. Commercial fillers show shears from slightly in excess of 10,000 seconds^{-1} to 100,000 seconds^{-1}. It is demonstrated that the combination of nozzle, piston, and filler geometry is such that each can become the point of critical shear, depending on the relative dimensions of the other parts.

IN THE pharmaceutical field, couette-type instrument rheology has been largely the standard of usage because any instrument with either a range of cups and/or bobs or of springs could be adapted to take the wide range of consistencies so characteristic of this discipline. The engineering profession and the polymer, plastics, and petroleum industries tended to adapt the classical Poiseuille capillary to their special needs by increasing the radius to tubes, and even pipes, and by using pressure to increase the driving force over that of gravity alone. Such an instrument, designed by Severs (1), is commercially available. Its use with pressurized dentifrice has been described elsewhere (2). This paper is intended to show some other possible adaptations for this useful and relatively inexpensive instrument.

As a rheometer alone it is flexible and covers a wide shear-rate range but this alone does not justify special note. It is, however, readily adaptable to give useful information in two fields where the couette instrument is not directly utilizable. The first of these is the study of thixotropic systems of slow recovery in which the mere process of loading the instrument with sample partially destroys the set. Many creams, pastes, and other semisolid systems fall into this category. Two types of measurement on such

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in castor oil is required. A simple dilution with methyl isobutyl ketone is the only sample preparation required.

4. Estrogenic hormones in tablets are extracted simply with N,N-dimethylformamide followed by dilution with methyl isobutyl ketone and filtration.

5. Accuracies in excess of 95% are obtained by simultaneously chromatographing replicates of standard and sample solutions on the same chromatogram.

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Technical Articles

Adaptation of Commercial Viscometers for Special Applications in Pharmaceutical Rheology II

Severs Extrusion Rheometer

By JOHN H. WOOD, GREGORY CATACALOS, and S. V. LIEBERMAN†

The Severs rheometer has been slightly modified to provide greater flexibility of use. Besides its normal use as a rheometer, it may be used to accept samples in collapsible tubes. Such samples undergo no change in thixotropic set before actual measurement. The apparatus can also be used to shear samples of up to 700-ml. volume in a manner comparable to a high-speed filler. The method of calibrating the filler shears is outlined. Commercial fillers show shears from slightly in excess of 10,000 seconds^{-1} to 100,000 seconds^{-1}. It is demonstrated that the combination of nozzle, piston, and filler geometry is such that each can become the point of critical shear, depending on the relative dimensions of the other parts.

IN THE pharmaceutical field, couette-type instrument rheology has been largely the standard of usage because any instrument with either a range of cups and/or bobs or of springs could be adapted to take the wide range of consistencies so characteristic of this discipline. The engineering profession and the polymer, plastics, and petroleum industries tended to adapt the classical Poiseuille capillary to their special needs by increasing the radius to tubes, and even pipes, and by using pressure to increase the driving force over that of gravity alone. Such an instrument, designed by Severs (1), is commercially available. Its use with pressurized dentifrice has been described elsewhere (2). This paper is intended to show some other possible adaptations for this useful and relatively inexpensive instrument.

As a rheometer alone it is flexible and covers a wide shear-rate range but this alone does not justify special note. It is, however, readily adaptable to give useful information in two fields where the couette instrument is not directly utilizable. The first of these is the study of thixotropic systems of slow recovery in which the mere process of loading the instrument with sample partially destroys the set. Many creams, pastes, and other semisolid systems fall into this category. Two types of measurement on such

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systems are then possible, conventional stress-shear rate determinations and the relative extrudibility of different formulations under constant stress. In this respect it becomes comparable to the modified De Waale plastometer described by Adler (3).

The second application, of considerable importance in industrial technology, is the application of the rheometer as a shear device to prepare samples of a known shear history for aging study. The properties of a thixotropic system of low recovery rate can be drastically changed by a subsequent shear hours or even days after original preparation. This generally is the commercial manufacturing and packaging procedure. Lotions and semisolid systems may be filled at varying times after manufacture. The filling shear will depend upon the type of equipment used, the rate of filling, and also for a given container rate, the container size filled. The calibration of a laboratory pseudofiller capable of encompassing such variables is then of practical value.

EXPERIMENTAL

The Rheometer.—The Castor-Severs extrusion rheometer, model A-100[1] was modified by the manufacturer to permit use over the range 0 to 200 p.s.i. A special sample tube of 700-ml. volume was supplied. It is 57 cm. long and 4 cm. internal diameter. This is approximately twice the length and volume of the usual rheometer. It has been found desirable to tee into the gauge line a manifold with a series of gauges for lower pressure ranges. Each is protected by a manual cut-off valve. (See Fig. 1.) Appropriate gauge calibrations were made primarily for internal self-consistency between gauges but also for absolute values. The instrument is supplied with nitrogen gas at 300 p.s.i. from cylinders. Flow times are measured with a foot pedal activated digital electric clock reading to 0.1 second. Density determinations are made in vessels suitable to the consistency of the material under investigation, fluid or semisolid. Three capillaries, 5.0 cm. long, are supplied. These have diameters of 0.6283, 0.3141, and 0.1556 cm.

Rheology of Thixotropes.—Because of the wide internal bore of the sample holder, paste-type samples can be examined if shears lower than those employed in loading can be ignored. Since such loading can be of quite low shear, this is frequently possible. More elegant, however, is the procedure of loading the sample upon preparation into conventional collapsible tubes of composition of lining compatible with the sample to be studied. Adapter plates were made by drilling a $^3/_8$-in. hole through an aluminum plate of the same dimensions as the capillary socket plate. The threaded portion of a tube cap was coaxially cemented over this hole with epoxy resin. For sample study, a tube was then uncapped, screwed into the plate, the end cut open, and the whole assembled into the rheometer with

[1] Burrell Corporation, Pittsburgh 19, Pa.

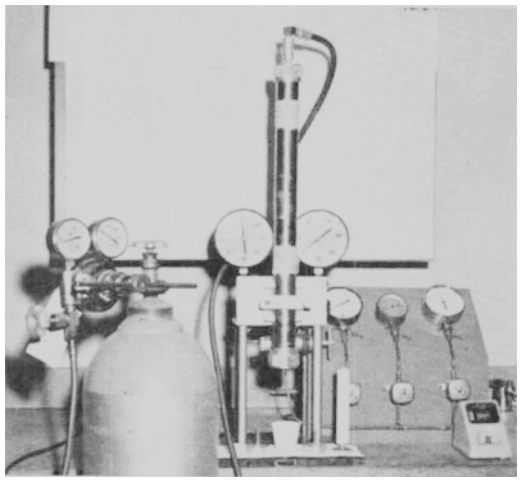

Fig. 1.—The modified Severs rheometer showing assembly and collapsible tube adapter.

the adapter plate immediately above the capillary plate. A light coat of vacuum grease is used between the two plates to prevent gas leakage. Conventional extrusion rheometry then follows. Alternately, by only using the adapter plate without the capillary, the pressure required for paste extrusion from a given sized tube may be determined for any desired delivery rates.

It has been found desirable, because of the large void space in the barrel, to prepressurize it while holding a finger over the end of the capillary. The timer is then foot activated as the finger is withdrawn. This is practical for pressures up to 100 p.s.i. The run is terminated instantly by releasing manually the extrusion pressure with the pressure release lever built into the apparatus. At the same time the timer is pedally stopped. The extrudate is caught and weighed in a tared container of suitable dimensions.

Rate of shear, D, is calculated from the equation

$$D = \frac{4Q}{\pi r^3} = \frac{4W}{\pi d r^3}$$

where Q is volume delivery per unit time, W the weight delivery per second, r is the radius of the capillary, and d the density of the extrudate.

The shearing stress, T, is given by

$$T = \frac{Pr}{2L}$$

where P is the driving pressure, and L is the length of capillary used.

Comparison values are shown in Fig. 2 for the measurement of a commercial dentifrice using each of the three capillaries in the extrusion rheometer. In addition, data were obtained from the Hercules Hi-Shear rheometer and from the D cup of the Drage Epprecht rheometer. In the latter case, the D cup was used alone and with each of the five sleeves available for it. The dentifrice system is highly thixotropic and is also pseudoplastic in rheology.

Pseudofilling.—The barrel is first charged with the sample. If the material under investigation is fluid, then the capillary is capped with a rubber plug; if the sample is semisolid then the bottom end is

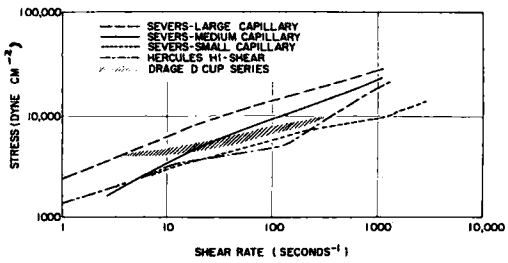

Fig. 2.—Rheograms run on an aged, strongly thixotropic, pseudoplastic dentifrice system with the Severs extrusion rheometer, the Hercules Hi-Shear rheometer, and the Drage Epprecht rheometer (D cup with sleeves).

capped with an end plate for loading and tamping, and the capillary is then put in place. Prepressurizing, as previously described, leads to the most consistent results. The appropriate volume, up to 700 ml. per charge, is then extruded at the desired rate, time of delivery and weight of extrudate being as before. For high rates of delivery, it is desirable to use two operators, one of whom notes the gauge pressure used and maintains its constancy. Under high flow rates, the diaphragm reducing system cannot deliver at sufficient rate to maintain the pressure preset at zero flow rate. If a series of points are obtained, then the resultant data also represent a high shear-rate rheogram. The samples made in this fashion are stored and monitored with passing time by low-shear measurements using our modified Brookfield system (4).

For the calibration of the equivalent shear of a filler, a full shear range set of samples are obtained by delayed filling of a thixotropic material whose "set" is partially broken by any shear at a time, days or hours, subsequent to making. Samples are simultaneously obtained from the filler. By using the exponential plot of aging behavior (5, 6), points of corresponding age and shear are compared directly for the rheometer prepared and the conventional filler samples (see Fig. 3). Equivalent shears of the filler are then noted as indicated in the graph by the solid triangles for points of identical rheological behavior. Usually the average of at least two periods of delayed fill and several periods of post-filling aging are obtained. Laboratory samples, pseudofilled equivalent to high-speed production filling, may thus be obtained for a wide range of shears to examine the effect of filling shears on subsequent rheological properties as part of a laboratory process study.

With a Colton 175–63 single nozzle filler, a study was made in the above manner of the resultant equivalent shear for two different operating speeds, two delivery piston sizes, and three filling nozzles, to permit an evaluation of whether the primary shear zone was in the piston or the orifice. Similarly two high-speed production fillers were also examined under their normal operating conditions.

RESULTS AND DISCUSSION

Rheology.—In the rheologic examination of highly thixotropic pseudoplastic systems, it is generally of sufficient utility to consider comparisons between samples made with only one measuring system. However, when two or more measuring systems are used, especially in a conventional couette system where the cup and bob ratios are changed, nonsuperimposable rheograms are obtained. Two phenomena, acting in opposition, are responsible for this. The first of these is the relaxation time of the thixotrope. The longer the time of applied shear, the greater the thinning that occurs. In a couette instrument the shear thinning at any shear rate is an integrated history of all the shear that has preceded. The measurement of a thixotropic index by the fall in torque with time is always complicated by the inability to measure the zero time value. In the capillary method, the length of the capillary sets the duration of the shear time for a given shear rate; therefore, a similar problem exists. However, the capillary is continuously using new sample so that a rheogram becomes independent of the rate at which increasing shear-rate points are taken.

The second consideration is correction for slippage either at the wall or internally. For the pseudoplastic system, which is not time dependent, Wilkinson (7) and others have outlined methods of calculation where data for various cup and bob ratios or different capillary radii are extrapolated to give measurements of torque and shear rate at the wall as the limiting case. From these extrapolations, true equations of flow may be derived.

However, for the thixotrope, the dimensions enter in a consideration of relaxation. The higher the shear-rate gradient, the greater the shear dependent thinning that occurs during a measurement. This is clearly shown in Fig. 2 for both types of measuring systems. In the couette type, as the Drage cup-to-bob ratio approached unity by the use of closer sleeves, the torque decreased for any given shear rate. For the Hercules, in which the

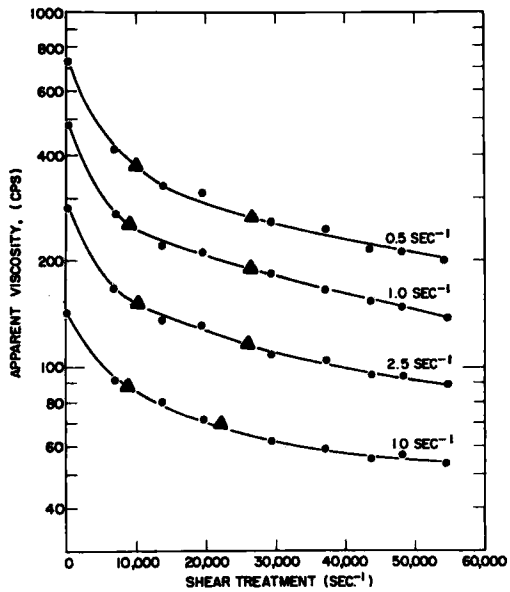

Fig. 3.—Apparent viscosities at different shear rates of a thixotropic lotion pseudofilled at various rates of shear, 2 days after manufacture. (Measurements were made after 7 days.) The triangular points represent equivalent behavior of material filled on two commercial fillers.

ratio is 0.95, the torque is even lower. Similarly decreasing radius of capillary resulted in lower torque for any given shear rate.

That the large capillary is capable of giving readings with minimal thixotropic breakdown is then evident from Fig. 2. Extrapolation procedures would indicate that the truly undisturbed readings are in excess of those found even with the large capillary.

The capillary type of measurement has another virtue. Most couettes are confined optimally to slightly over two decades of shear. In practice a given capillary is useful for almost four decades of shear rate.

Using the collapsible tube system, one restriction is necessary. The neck size must be such that a negligible portion of the shear occurs there relative to the measuring capillary.

The case shown here, the highly thixotropic dentifrice system, shows the worst possible disagreement between instruments. Normally the agreement is much more consistent and the dependency on dimensions far less critical. For nonthixotropic pseudoplastic substances, excellent agreement between measuring systems of the capillary and couette type is the general rule.

Pseudofilling.—In Fig. 3, for a cosmetic antiperspirant lotion, we see the agreement obtainable for four shear rates of measurement for the apparent shear exerted by a filler as determined by the rheological properties measured at a later date. Similar agreement was obtained between other test situations of different ages before filling and also different ages for post examination. In this way we concluded that one production filler filling 1-oz. bottles exerted a shear equivalent to approximately 25,000 seconds^{-1} while another filling 1$^1/_2$-oz. bottles gave only 12,000 seconds^{-1}, thus emphasizing the role played by filler dimensions. This was subsequently shown in detail with the Colton filler.

An examination of the equivalent shear of the Colton filler operating at full speed with full and partial stroke is given in Table I. For reduced speeds, for those nozzle, stroke, piston combinations examined the apparent shear was proportional to the delivery rate. Using the small piston, with either full or partial stroke, the primary shear zone appears to be in the region from the piston to the orifice for the large and medium orifices. The small orifice, however, either contributes to or becomes the most significant shear zone. With the large piston, it is evident that the medium orifice now contributes significantly to the shear imposed, and it is not clear whether this applied to the large orifice too. Admittedly full speed, full stroke, filling is not a commercial practicality, but these data do demonstrate the role filler shear can play in subsequently important rheological properties. The data of Table I also emphasize that filling conditions set what part of the machine becomes the source of primary shear.

TABLE I.—EFFECT OF MACHINE VARIABLES ON APPARENT SHEAR OF A COLTON FILLER

		Small Piston Delivery		Large Piston Delivery	
Stroke	Nozzle	Rate, Gm./sec.	Shear, sec.$^{-1}$	Rate, Gm./sec.	Shear, sec.$^{-1}$
Full	Large	58	37,000	143[a]	53,000
	Medium	58	37,000	171[a]	105,000
	Small	58	47,000	142[a]	112,000
Partial	Large	36	29,000	93	16,000
	Medium	37	28,000	92	54,000
	Small	38	36,000	85	78,000

[a] Possible sample loss due to splashing at such high flow rates.

Only an extrusion type rheometer is practically capable of applying shears in the range found for commercial fillers. Being able to collect samples that have been subjected to known shear rates in quantities suitable for study is a distinct advantage to the modified Severs described here.

SUMMARY

The commercially available Severs rheometer is shown to be applicable directly, and by minor adaptation, to rheological problems of concern in the properties of lotions and semisolids. By the use of samples stored in collapsible tubes of suitable composition almost "undisturbed" rheological measurements of thixotropes may be made.

The bulk preparation of samples prepared under various known rates of shear permits calibration of the apparent shear of commercial filling equipment and the study of shear dependency in the laboratory. The shear rates for filling may range from 10,000 sec.$^{-1}$ to 100,000 sec.$^{-1}$. The critical dimensions to shear in a commercial piston filler vary with the piston and orifice tube sizes used.

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Compressed Coated Tablets I

Measurement and Factors Influencing Core Centration

By LEON LACHMAN, PETER P. SPEISER†, and HANNA D. SYLWESTROWICZ

A novel and accurate procedure for measuring the horizontal and vertical centration of tablet core in compressed coated tablets is described. This method of measurement permits the determination of core dislocation along and across the axis of turntable movement. The influence of size distribution of the coating granulation on core expansion and centration was studied. Results are presented for compressed coated tablets processed under similar operating characteristics on the Manesty and Kilian machines. A statistical treatment of the data was performed to permit a more meaningful interpretation and comparison of the results within one machine as well as between machines. This method for measuring core centration permits the determination of the size distribution of granulation necessary to give optimal centration.

THE PAST several years have seen the principle of coating tablets by compression meet with wide acceptance and enthusiasm in the pharmaceutical industry. This is evidenced by the numerous compressed coated tablets on the market today.

Literature reports relative to dry coating also began to appear during this time. Linde (1) and Windheuser and Cooper (2) reported on the physical properties required for core and coating granulations to give suitable compressed coated tablets. Wolff (3) presented coating and core formulations adequate for use in compression coating. Klump, *et al.* (4), have shown that the process of dry coating can be used to hide the bitter taste of active medicaments, to eliminate discoloration, and to improve the stability of active ingredients. Core formulations intended for sustained action dry coated tablets have been described by Cooper and Windheuser (5). Several reports have recently appeared in the literature pertaining to the application of an enteric coating to a core by compression methods (6–9).

It is evident from the above that considerable research has gone into the development of suitable formulas for compression coating. On the other hand, there appears to be little work reported relative to accurate methods for measuring horizontal and vertical centration of cores in compressed coated tablets. Weinstein (10) made an attempt in this direction by employing a roentgenographic technique. This consisted of including a small concentration of elemental iron in the core formulation and subsequently developing a radiographic plate showing the contrast between core and coating. Using this method the author was only able to present a general picture of core dislocation in the horizontal plane and cocking in the vertical plane.

In this report an accurate procedure for measuring horizontal and vertical centration of tablet cores in compressed coated tablets will be described. The method to be illustrated permits the determination of core dislocation in the horizontal plane, both parallel and at right angles to turntable movement, as well as maximum dislocation. In addition, it is possible to determine cocking in the vertical plane along the same three vectors and also core displacement up or down along the vertical axis. The utility of this method for determining the optimal size distribution of a coating granulation to give the most satisfactory core centration is demonstrated. This was done for the Manesty and Kilian dry coating machines using one formula granulation which was fractionated to give four granulations, each having a different size granule as its major component.

EXPERIMENTAL

Core Tablets

The core tablets were prepared according to the following formula and procedure:

Material and Formula.—Lactose U.S.P., 10.000 Kg.; wheat starch, 2.750 Kg.; Aerosil Compositum,[1] 0.750 Kg.; gelatin U.S.P., 0.250 Kg.; stearic acid, spray dried, 0.625 Kg.; charcoal, 0.125 Kg.; talcum U.S.P., 0.500 Kg.; and purified water, *q.s.*

Procedure.—The granulation was prepared according to customary wet granulating techniques using gelatin solution as the granulating agent. The wet granulation was dried at 40° in a circulating

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[1] Aerosil Compositum is composed of 85% colloidal silica and 15% hydrolyzed starch.

air oven to a moisture content of 1.5%. The dried granulation was screened through a No. 12 mesh screen in a Fitzpatrick model D comminuting machine. Into this granulation were mixed the charcoal and stearic acid, which were previously passed through a No. 40 mesh screen.

Table I shows the sieve analysis for a representative sample of this granulation.

TABLE I.—SIEVE ANALYSIS OF CORE GRANULATION

Sieve Number	Per Cent on Screen
8	60.5
12	34.5
20	4.0
30	0.5
50	0.5

Coating Granulation

Material and Formula.—This granulation was prepared according to the procedure described under the core tablets. The following was the formula used: lactose U.S.P., 16.000 Kg.; Aerosil Compositum, 1.500 Kg.; gelatin U.S.P., 0.500 Kg.; wheat starch, 2.500 Kg.; arrowroot starch, 2.500 Kg.; talcum U.S.P., 1.000 Kg.; stearic acid, spray dried, 1.000 Kg.; and purified water, *q.s.*

The granulation was then divided into four parts and each part sieved through different size screens to give granulations of different size distribution. The sieve analysis data for these four batches of granulation are presented in Table II.

TABLE II.—SIEVE ANALYSIS OF THE FOUR COATING GRANULATIONS

Granulation Fraction	Per Cent on Screen					Per Cent Through Screen
	No. 8	No. 12	No. 20	No. 30	No. 50	No. 100
8	8.0	68.5	15.5	2.0	5.0	1.0
35	0.0	26.0	43.5	6.0	18.5	6.0
50	0.0	0.2	13.5	9.0	56.0	21.3
100	0.0	0.0	0.0	0.1	15.5	84.4

Preparation of Compressed Coated Tablets on Manesty DryCota and Kilian Prescoter.—The core tablets used for both machines were compressed on the core side of the Manesty DryCota model 350 at a rate of 18,000 tablets per hour. They weighed 150 mg., were 7 mm. in diameter (0.276 inch), and had a radius of curvature of 13 mm. The dry coated tablets produced weighed 400 mg., were 10 mm. in diameter (0.394 inch), and had a radius of curvature of 18 mm.

The Manesty machine is a 16-station press, while the Kilian machine has 20 stations. The die table of the Manesty machine traveled at 18.7 r.p.m. to produce 18,000 tablets per hour, while the die table on the Kilian machine traveled at a rate of 15 r.p.m. to produce the same number of tablets per hour.

Influence of Coating Size Distribution on Core Centration.—In order to determine the influence of particle size on the centering of the core in the finished compressed coated tablets, the particle size distribution of the core granulation was kept constant, while that of the coating granulation varied. Compressed coated tablets were prepared on the Manesty and Kilian machines using the four coating granulation fractions previously described. After the machine was regulated with regard to weight uniformity for each coating granulation, samples of 100 tablets were taken initially and then every 10 minutes for an hour without adjusting the machine during the hour's run. From each of these 100 compressed coated tablets, 10 were taken at random for determining horizontal centration and 20 were taken at random for vertical centration measurements. Of these 20 tablets, 10 tablets were cut crosswise to and the other ten tablets were cut along the axis of die table movement.

Core Centration Measurements

Horizontal Centration.—Figure 1 shows a schematic diagram of a die table and gives the direction of rotation of the die table used for compressing the coating granulation around the core for both the Manesty and Kilian machines. As the coated tablet begins to leave the bore of the die it is marked on the outside of the tablet cylinder with a special marking pen attached to the machine. This takes place just before the take-off point. Marking the tablets in this manner identifies the tablet position with regard to the direction of die table movement. It is then possible to measure the core dislocation along and across the axis of die table movement as illustrated in Fig. 1. In addition, by further calculation it is also possible to estimate the maximum dislocation of the core within the tablet.

In order to permit an accurate measure of core dislocation, tablets were cut along the horizontal surface to a definite depth with the device shown in Fig. 2. Then fivefold magnification photographs were taken, an example of which is shown in Fig. 3. Centration measurements were made using these photographs.

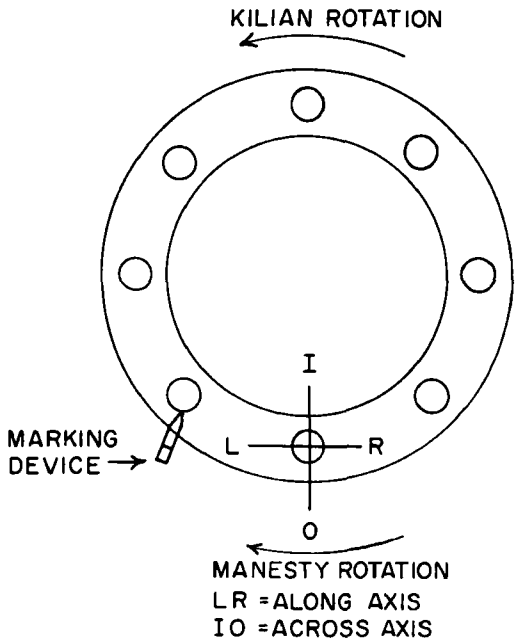


Fig. 1.—Schematic diagram of die table showing marking device attachment and direction of rotation for Manesty and Kilian machines.

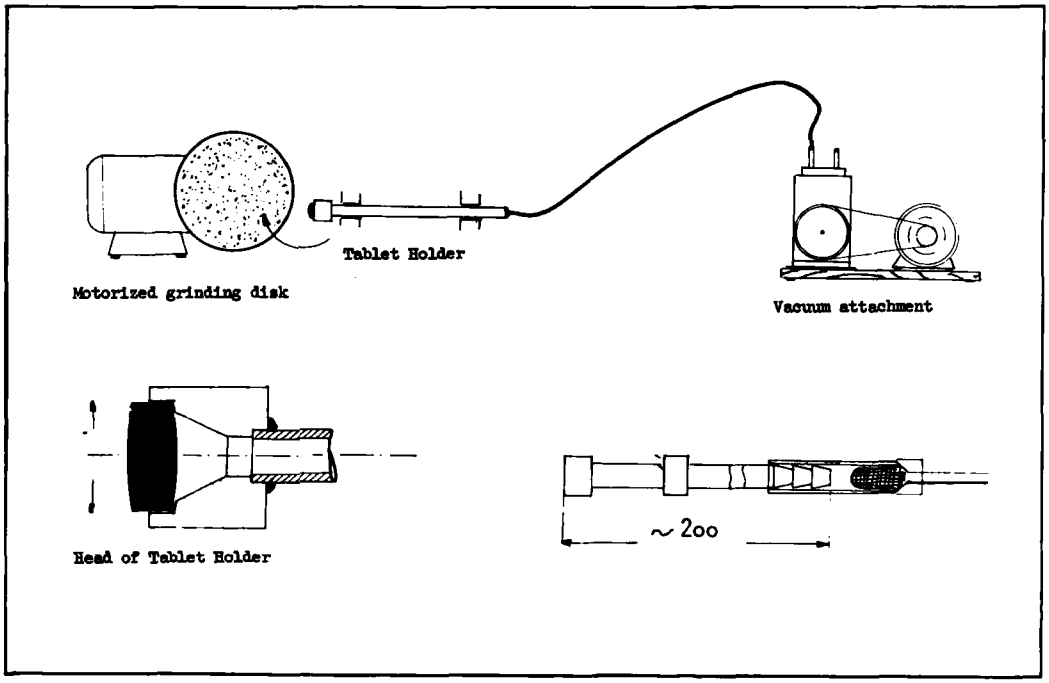


Fig. 2—Tablet cutting device.

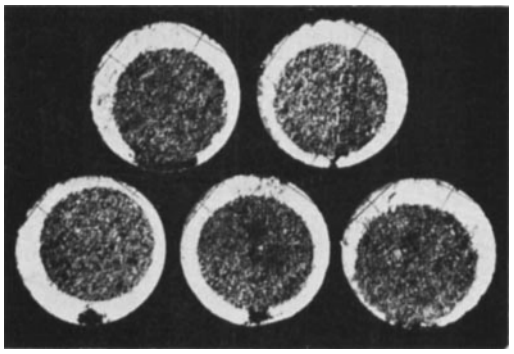


Fig. 3.—A five-fold magnification photograph of horizontal cut tablets.

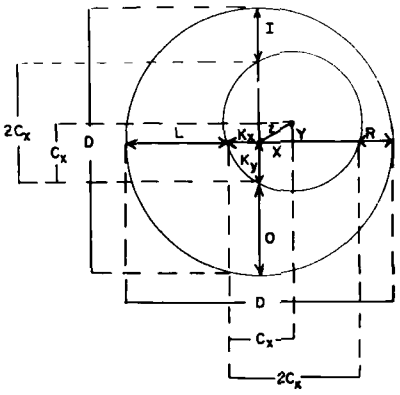
In the past, attempts to measure core centration consisted at best of determining the maximum dislocation of the core in the tablet. Only in the ideal situation, where core dislocation has taken place on the diameter of the tablet along the axis of die table movement, can direct measurements be made of core dislocation by determining the difference between the distance at the left and right between the core and coating along the diameter of the tablet. This ideal situation is seldom observed. Instead, the center of the core is dislocated from the diameter of the tablet along and across the axis of die table movement or in both directions. In this situation if any attempt is made to use the difference between the left and right side of the core edge to the coating edge, incorrect results would be obtained in the estimation of core dislocation. To accurately measure core dislocation along the horizontal plane, the geometry of the tablet was taken into consideration and equations derived to accurately determine centration "along axis" and "across axis" of die table movement and the maximum dislocation. Figure 4 represents a graphic model of the core dislocated from the center of the tablet along and across the horizontal plane. The resulting right triangle having the sides X, Y, and Z, drawn from the center of the tablet to the center of the core, was used to derive the equations needed to measure core dislocation. The equations needed to determine "along axis" dislocation of center of core from center of tablet (X), "across axis" dislocation of center of core from center of tablet (Y), and maximum dislocation of center of core from center of tablet (Z) are as follows:

$$X = \frac{L - R}{2} \qquad Y = \frac{O - I}{2} \qquad Z = \sqrt{X^2 + Y^2}$$

The symbols used in the derivation of the equations from Fig. 4 are defined as follows: D = diameter of tablet; R = distance between core and coating on the right side; L = distance between core and coating on the left side; and R and L are measurements for dislocation along the axis of turntable movement. O = distance between core and coating on the outside; I = distance between core and coating on the inside; and O and I are measurements for dislocation across the axis of turntable movement.

Now if: $X = 0$, the core is not dislocated along axis of turntable movement; $X > 0$, the core is dislocated to the right; $X < 0$, the core is dislocated to the left; $Y = 0$, the core is not dislocated across axis of turntable movement; $Y < 0$, the core is dislocated toward the outside, and if $Y > 0$, the core is dislocated toward the inside.

It is evident from the above equations that it is only necessary to know the values for L, R, I, and O to be able to determine core dislocation in the three directions X, Y, and Z. In order to measure



Derivation of Formulas for x, y and z

x	y	z
Since $x = C_x - K_x$	Since $y = C_y - K_y$	Since $z^2 = x^2 + y^2$
$K_x = \frac{D}{2} - L$	$K_y = \frac{D}{2} - O$	$z = \sqrt{x^2 + y^2}$
$2C_x = D-(R+L)$	$2C_y = D - (O+I)$	
$C_x = \frac{D-(R+L)}{2}$	$C_y = \frac{D - (O+I)}{2}$	
Then	Then	
$x = \frac{D-R-L}{2} - \frac{D}{2} + L$	$y = \frac{D-O-I}{2} - \frac{D}{2} + O$	
$x = -\frac{R}{2} - \frac{L}{2} + L$	$y = \frac{O}{2} - \frac{I}{2} + O$	
$x = \frac{L-R}{2}$	$y = \frac{O-I}{2}$	

Fig. 4.—A graphic illustration of core dislocation along horizontal plane and derivation of equations for "along axis," "across axis," and maximum dislocation of center of core from center of tablet.

these values the exact center of the tablet was determined by drawing two cords along the edge of the coating and taking the intersection of the perpendicular bisectors of these cords as the center.

Horizontal core centration was determined by measuring *R*, *L*, *I*, and *O* on 70 tablets taken from each of the four coating granulations after compression on both the Manesty and Kilian machines.

Vertical Centration.—Since the tablets were marked as they came out of the die cavity before the takeoff point, it was possible to measure core off-centering "along" and "across" the axis of die table movement by cutting the tablets along *L*, *R*, *I*, or *O*, respectively, as shown in Fig. 1. This is more adequately illustrated in Fig. 5, where the A portion shows the cutting of the tablet "across" die table movement and the B, "along" the axis of die table movement. Tablets so cut were photographed under fivefold magnification as shown in Fig. 6 and measurements for core centration were made from these photographs.

Off-centering of the core in the vertical plane can take place either by (*a*) tilting or (*b*) displacement of the center of the core up or down along the center axis of the tablet as illustrated by portions B and C, respectively, of Fig. 7. If the distances *AB* and *CD* as shown in portion A of Fig. 7 were measured, incorrect data would result. This is due to the fact that if the core shifted to the left or right of the center axis it would show up by this method of measurement. Since this dislocation is representative of off-centering in the horizontal plane, it was measured earlier. However, by measuring the dislocation along *HG* and *EF* a true indication could be obtained of core off-centering in the vertical plane, thus eliminating the interference in measurement due to horizontal dislocation. Accordingly, this technique of measuring was used to determine tilting, as illustrated by portion *B* of Fig. 7 and up and down displacement, as shown by portion C of Fig. 7.

By measuring the distances *L* and *L'* and *R* and *R'*, *O* and *O'*, and *I* and *I'*, as illustrated in portion B of Fig. 7, it is possible to determine tilt "along axis" (T), tilt "across axis" (T_l), maximum tilt (T_m), displacement "along axis" (D), and displacement "across axis" (D_l). The equations used to determine T and T_l are as follows

$$T = \frac{(R - L) + (L' - R')}{2}$$

$$T_l = \frac{(O - I) + (I' - O')}{2}$$

T and T_l could be determined from $R - L$ and $O - I$ alone, but by using the means of $(R - L)$ and $(L' - R')$ or $(O - I)$ and $(I' - O')$ more precise data are obtained by minimizing the error of measurements.

Now if: $T = 0$, no tilt takes place along axis of turntable movement; $T < 0$, the left edge of the core is tilted up; $T > 0$, the right edge of the core is tilted up; $T_l = 0$, no tilt takes place across axis of turntable movement; $T_l < 0$, the inside edge of

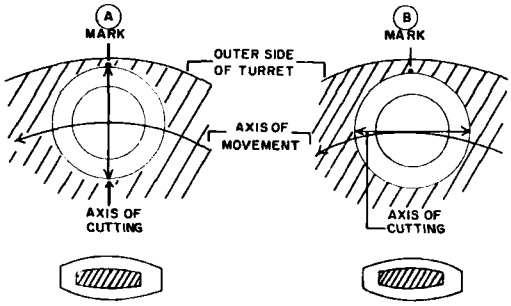


Fig. 5.—A schematic diagram showing the cutting of tablets vertically along and across the axis of die table movement.

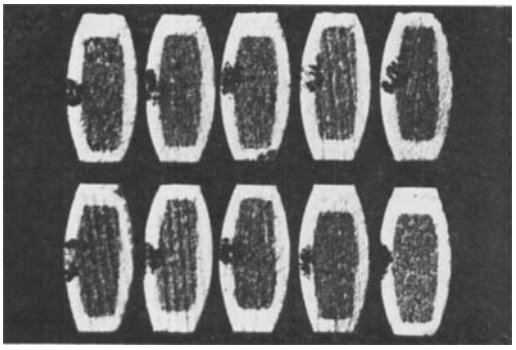


Fig. 6.—A five-fold magnification photograph of tablets cut along vertical plane.

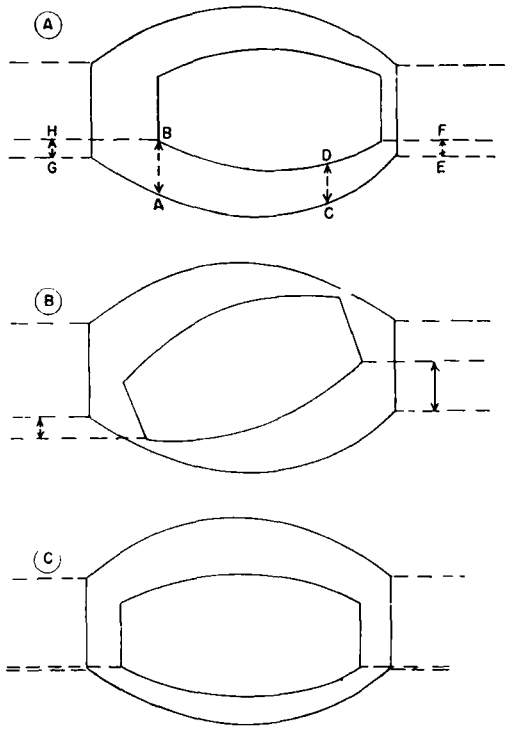


Fig. 7.—A graphical illustration indicating off-centering of core in the vertical plane of the tablet. (A) = horizontal dislocation, B = tilting, C = displacement along vertical plane. Lines AB and CD indicate improper distances to measure, while HG and EF are the correct distances to determine tilt and displacement in vertical plane.

core is tilted up; and if $T_l > 0$, the outside edge of core is tilted up.

To assist in the derivation of Tm, a spatial view of a tilted core is presented in Fig. 8. The solid line shows the tilted core and the dotted line its projection in space. The angle α is between the tilted core and its projection and opposite to T, Tl, and Tm. The triangles are right triangles. The letter ρ is equal to the radius of the core. From this figure it would appear that the values of ρ are different along the various radii, but this is only an optical illusion.

Side views of the right triangles in the spatial diagram give the projection shown by portion A of Fig. 9, and an imaginary top surface view of the tilted core is shown in portion B of Fig. 9.

From the right triangles in portion A of Fig. 9, the following equations can be derived

$$\frac{Tm}{Km} = \frac{T}{K} = \frac{T_l}{K_l} \qquad \text{(Eq. 1)}$$

or

$$\begin{aligned} TmK &= TKm \\ TmK_l &= T_lKm \end{aligned} \qquad \text{(Eq. 2)}$$

In portion B of Fig. 9, β is the same angle for both triangles because they are right triangles and the line across axis is perpendicular to the line along axis. Therefore

$$Km = \rho \qquad \text{(Eq. 3)}$$

$$K_l = \rho \cdot \cos\beta \qquad \text{(Eq. 4)}$$

$$K = \rho \cdot \sin\beta \qquad \text{(Eq. 5)}$$

By substituting Eqs. 3, 4, and 5 into Eqs. 1 and 2, we obtain the derivation for the formula to be used to measure maximum tilt (Tm)

$$\begin{aligned} Tm\,\rho \sin\beta &= T\,\rho & Tm\,\rho\cos\beta &= T_l\,\rho \\ T &= Tm \sin\beta & T_l &= Tm\cos\beta \\ T^2 &= Tm^2\sin^2\beta & T_l^2 &= Tm^2\cos^2\beta \\ T^2 + T_l^2 &= T^2m\,(\sin^2\beta + \cos^2\beta) \end{aligned}$$

since $\sin^2\beta + \cos^2\beta = 1$, then $T^2 + T_l^2 = Tm^2$ and $Tm = \sqrt{T^2 + T_l^2}$.

In the case where $T \neq 0$ and $T_l \pm 0$, Tm should be determined from the above formula. Such a situation means that the axis of maximum tilt is located somewhere between the "along axis" and the "across axis" of tablet. If the axis of maximum tilt lies on the "along axis" of tablet, then $T = 0$ and $Tm = T_l$. In the case where the maximum tilt lies on the "across axis" of tablet, $T_l = O$ and $Tm = T$.

The equations used to determine core displacement "along axis," D, and core displacement "across axis," D_l, are

$$D = \frac{(R + L) - (R' + L')}{4}$$

$$D_l = \frac{(O + I) - (O' + I')}{4}$$

These equations were derived in the following manner: If the core is displaced by the amount D upward

$$\left.\begin{matrix} R \text{ is increased by } D \\ L \text{ is increased by } D \\ R' \text{ is decreased by } D \\ L' \text{ is decreased by } D \end{matrix}\right\} (R + L) - (R' + L') = 4D$$

Therefore

$$D = \frac{(R + L) - (R' + L')}{4}$$

The derivation for D_l is analogous to D. Now if: $D = 0$, no displacement takes place along the axis; $D < 0$, downward displacement takes place along the axis; $D > 0$, upward displacement takes place along the axis; $D_l = 0$, no displacement takes place across the axis; $D_l < 0$, downward displacement takes place across the axis; and if $D_l > 0$, upward displacement takes place across the axis.

In measuring core tilting and displacement in the

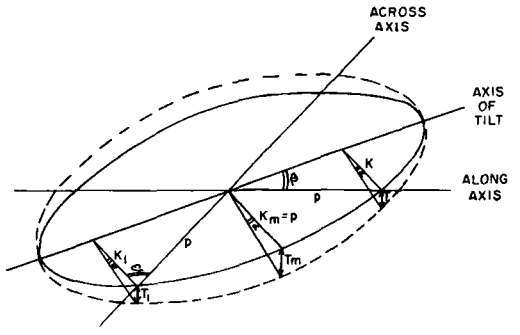


Fig. 8.—A spatial view of tilted core.

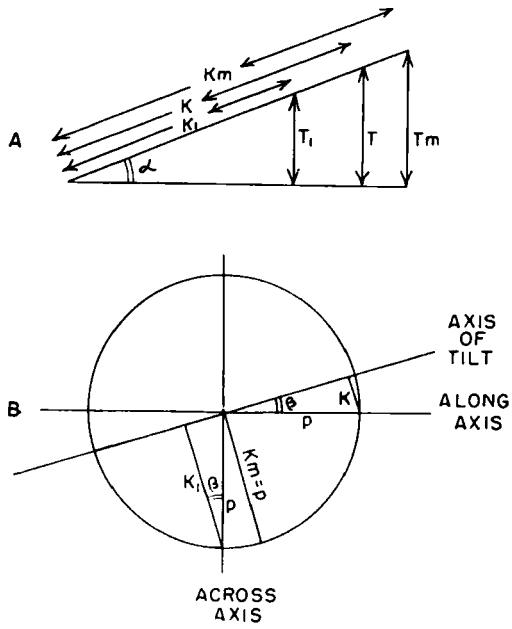


Fig. 9.—Side views of triangles of the spatial diagram A and top view of core shown in spatial diagram B.

vertical plane, measurements for R, R', L, L', O, O', and I, I' were taken for each of the four coating granulations on 70 tablets (10 tablets per each of seven time intervals) for both the Manesty and Kilian machines.

Core Expansion.—Since experience has indicated that the core tablet undergoes expansion in the lateral direction when compressed a second time in the bed of coating granulation, measurements were made of core diameter for tablets produced with the four coating granulations on each machine. The center of the core was obtained in the same manner as was described for determining the center of the tablet. Once the center of the core was obtained, the diameter measurements were readily made. These were done on the fivefold magnification photographs of the horizontally cut tablets.

Statistical Analysis of Data.—The Manesty and Kilian machines were analyzed separately and then compared for each of the following nine parameters for core centration: *1.* dislocation along axis (X), *2.* dislocation across axis (Y), *3.* maximum dislocation (Z), *4.* tilt along axis (T), *5.* tilt across axis (T_i), *6.* maximum tilt (Tm), *7.* displacement along axis (D), *8.* displacement across axis (D_i); and *9.* core expansion (E).

The purpose of this analysis was to determine whether the dislocations, tilts, and displacements were significant (comparison with the theoretical value of zero) and if they differed significantly among themselves. Additional evaluations were performed to determine if there were significant differences between the "along axis" and "across axis" dislocations and tilts.

As methods of analysis, the F test and analysis of variance were employed. In order to specify which of the means are significantly different from one another and from the theoretical zero value, a test suggested by Professor A. P. Dempster of Harvard University (11) was employed. This test is an alternative to the D. B. Duncan multiple range test (12) and is based on tables of F distribution and on the so-called 95% simultaneous confidence statement about true (unknown) means.

For the evaluation of maximum dislocation (Z) and maximum tilt (Tm) it was necessary to estimate and subtract the variance due to bias of measurement because the formulas for Z and Tm contain squared units of measurement. This bias correction was done for each granulation on each of the parameters Tm and Z.

RESULTS AND DISCUSSION

Horizontal Centration

The results obtained from the analysis of core dislocation of tablets prepared on the Manesty and Kilian machines are summarized in Tables III and

Table III.—Measurements of Core Dislocations on Horizontal Cuts for Tablets Prepared on the Manesty Machine[a]

Dislocation Along Axis (X)					
Granulation fraction	50	35	8	100	
Means of X	0.0685	0.0814	0.1338	0.2478	
Level of significance with theoretical 0	5%	1%	0.1%	0.1%	
Comparison between granulations					1% level
Dislocation Across Axis (Y)					
Granulation fraction	8	35	50	100	
Means of Y	−0.1505	−0.1564	−0.1957	−0.2441	
Level of significance with theoretical 0	0.1%	0.1%	0.1%	0.1%	
Comparison between granulations					5% level
Maximum Dislocation (Z)					
Granulation fraction	35	50	8	100	
Means of Z	0.2283	0.2502	0.2542	0.4122	
Level of significance with theoretical 0	Not	1%	1%	0.1%	
Comparison between granulations					0.1% level
Difference Between Absolute Values of Y and X					
Granulation fraction	100	8	35	50	
$\lvert Y\rvert - \lvert X\rvert$	0.0036	0.0167	0.0750	0.1272	
Level of significant difference	Not	Not	Not	0.1%	

[a] Any two means not underscored by the same line are significantly different while those underscored by the same line do not differ significantly.

TABLE IV.—MEASUREMENTS OF CORE DISLOCATIONS ON HORIZONTAL CUTS FOR TABLETS PREPARED ON THE KILIAN MACHINE[a]

	Dislocation Along Axis (X)				
Granulation fraction	8	100	35	50	
Means of X	−0.0994	−0.1078	−0.1550	−0.3071	
Level of significance with theoretical 0	Not	Not	1%	0.1%	
Comparison between granulations					5% level
	Dislocation Across Axis (Y)				
Granulation fraction	8	100	35	50	
Means of Y	0.0080	0.0192	−0.2314	−0.2757	
Level of significance with theoretical 0	Not	Not	0.1%	0.1%	
Comparison between granulations					1% level
	Maximum Dislocation (Z)				
Granulation fraction	100	8	35	50	
Means of Z	0.1676	0.3795	0.4890	0.5255	
Level of significance with theoretical 0	Not	0.1%	0.1%	0.1%	
Comparison between granulations					0.1% level
					1% level
	Difference Between Absolute Values of Y and X				
Granulation fraction	8	50	35	100	
$\lvert Y\rvert - \lvert X\rvert$	0.0039	0.0314	0.0735	0.0871	
Level of significant difference	Not	Not	Not	Not	

[a] Any two means not underscored by the same line are significantly different while those underscored by the same line do not differ significantly.

IV, respectively. The significant dislocations shown in both tables are graphically illustrated in Fig. 10.

Manesty Machine.—The tablets prepared with all four different size distribution granulations show the core to be significantly dislocated toward the right ($+X$) and to the outside ($-Y$) of the compressed coated tablet. The tablets prepared with granulation fraction 100 exhibit cores that are significantly more dislocated than the cores in the tablets prepared from the other three granulation fractions, which do not differ in dislocation among themselves.

Kilian Machine.—For this machine only the tablets prepared with granulation fractions 35 and 50 show cores that are dislocated toward the left ($-X$) and to the outside ($-Y$) of the compressed coated tablets and these dislocations differ significantly from the tablets prepared with the other two granulation fractions.

The difference between the absolute mean values for "across axis" and "along axis" core dislocations

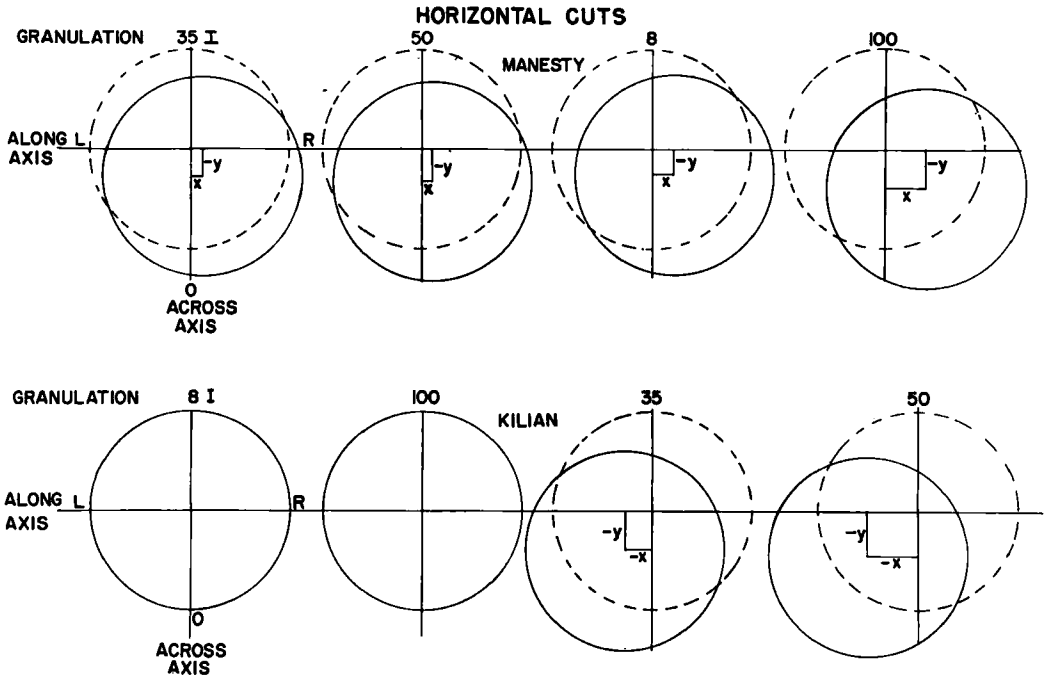


Fig. 10.—A graphic illustration of the influence of coating granulation size on core dislocation along the horizontal plane for tablets produced on the Manesty and Kilian machines. Only statistically significant dislocations are shown.

TABLE V.—CORE EXPANSION FOR MANESTY AND KILIAN MACHINES[a]

	Manesty				
Granulation fraction	100	50	35	8	
Means of expansion	7.44	7.62	7.73	7.78	
Comparison between granulations	——	————	————	——	1% level
	Kilian				
Granulation fraction	100	50	8	35	
Means of expansion	7.42	7.63	7.61	7.82	
Comparison between granulations	——	————	————	——	0.1% level

[a] Any two means not underscored by the same line are significantly different while those underscored by the same line do not differ significantly.

was proved significant only for the tablets prepared with granulation fraction 50 for the Manesty machine. This would mean that the cores are more dislocated toward the outside than toward the right position in the compressed coated tablet.

In analyzing the core dislocations of tablets prepared on the Manesty and Kilian machines, it was found that no conclusions could be drawn simultaneously for both machines. Consequently, the results for each machine must be reported separately.

Core Expansion

The results obtained for the Manesty and Kilian machines are presented in Table V. For both machines the core expansion in the tablets prepared with granulation fraction 100 is significantly smaller than that obtained with the other three granulation fractions.

Since the data for both machines, when analyzed simultaneously, showed significant interaction between machine and granulation fraction, the conclusions concerning core expansion for the granulation fractions must be drawn independently for each machine.

Vertical Centration

The data obtained from the analysis of tilt and displacement of core for the tablets prepared on the Manesty machine are summarized in Tables VI and VII and those for the Kilian machine in Tables VIII and IX. In Figs. 11 and 12 the tilt and displacement data found to be statistically significant are graphically presented for the Manesty and Kilian machines, respectively.

Manesty Machine.—From the mean values of tilt in the three directions, it seems that the tablets prepared with coating granulation fraction 8 show a consistent tendency for least tilting of core. On the other hand, the mean values of core displacement "along" and "across axis" show that the tablets prepared with granulation fraction 100 give the least displacement "along axis" and those prepared with granulation fraction 35 give the least displacement "across axis." But in each case, granulation fraction 8 results in tablets having cores exhibiting the most significant displacement. Where the displacement is significant, it is always in the upward direction for both "along" and "across axis."

Kilian Machine.—It is apparent from the data that the tilting of core has the tendency to take place in the upward direction at the left side of the tablet for the "along axis" and in the upward direction at the inside of the tablet for the "across axis" cuts. From the mean values of tilt in the three directions it is evident that the tablets prepared with granulation fractions 8 and 100 show the least tilting of core. However, for core displacement, "along" and "across" die table movement, the mean values obtained indicate that the tablets

TABLE VI.—MEASUREMENT OF CORE TILT ON VERTICAL CUTS FOR TABLETS PRODUCED ON THE MANESTY MACHINE[a]

	Tilt Along Axis (T)				
Granulations	8	35	50	100	
Means of tilt	0.0207	0.0395	0.0750	0.1135	
Level of significance of tilt	Not	Not	Not	1%	
Comparison between granulations	————				Not signif.
	Tilt Across Axis (T_i)				
Granulations	8	35	50	100	
Means of tilt	−0.0001	0.0330	−0.0367	−0.1613	
Level of significance of tilt	Not	Not	Not	0.1%	
Comparison between granulations	————			————	1% level
	Maximum Tilt (Tm)				
Granulations	8	35	50	100	
Means of tilt	0.0718	0.0907	0.1157	0.2353	
Level of significance of tilt	Not	Not	Not	0.1%	
Comparison between granulations	————			————	5% level
	Difference Between Tilt Across Axis and Along Axis				
Granulations	50	35	8	100	
$\lvert T_i \rvert - \lvert T \rvert$	−0.0692	−0.0123	0.0011	0.0563	
Level of significance	Not	Not	Not	Not	

[a] Any two means not underscored by the same line are significantly different while those underscored by the same line do not differ significantly.

TABLE VII.—MEASUREMENT OF CORE DISPLACEMENT ON VERTICAL CUTS FOR TABLETS PRODUCED ON THE MANESTY MACHINE[a]

	Displacement Along Axis (D)				
Granulations	35	100	50	8	
Means of displacement	−0.0620	0.0586	0.0717	0.1417	
Level of significance of displacement	Not	Not	Not	1%	
Comparison between granulations	———			———	1% level
	Displacement Across Axis (D_l)				
Granulations	35	100	50	8	
Means of displacement	0.0014	0.0775	0.1113	0.2480	
Level of significance of displacement	Not	1%	0.1%	0.1%	
Comparison between granulations	———	———		———	1% level
	———		———		5% level

[a] Any two means not underscored by the same line are significantly different while those underscored by the same line do not differ significantly.

TABLE VIII.—MEASUREMENT OF CORE TILT ON VERTICAL CUTS FOR TABLETS PRODUCED ON THE KILIAN MACHINE[a]

	Tilt Along Axis (T)				
Granulations	100	8	35	50	
Means of tilt	−0.0042	−0.0282	−0.0317	−0.0450	
Level of significance of tilt	Not	Not	Not	Not	
Comparison between granulations	———				Not signif.
	Tilt Across Axis (T_l)				
Granulations	100	8	35	50	
Means of tilt	0.0326	−0.0015	−0.0564	−0.0842	
Level of significance of tilt	Not	Not	5%	0.1%	
Comparison between granulations	———		———		1% level
		———		———	5% level
	Maximum Tilt (Tm)				
Granulations	100	8	50	35	
Means of tilt	0	0	0.0890	0.1029	
Level of significance of tilt	Not	Not	Not	5%	
Comparison between granulations	———				Not signif.
	Difference Between Tilt Across Axis and Along Axis				
Granulations	100	8	35	50	
$\lvert T_l \rvert - \lvert T \rvert$	0.0155	0.0093	0.0047	0.0412	
Level of significance	Not	Not	Not	Not	

[a] Any two means not underscored by the same line are significantly different while those underscored by the same line do not differ significantly.

TABLE IX.— MEASUREMENT OF CORE DISPLACEMENT ON VERTICAL CUTS FOR TABLETS PRODUCED ON THE KILIAN MACHINE[a]

	Displacement Along Axis (D)				
Granulations	35	50	100	8	
Means of displacement	−0.0400	−0.0771	−0.0800	−0.1082	
Level of significance of displacement	Not	0.1%	0.1%	0.1%	
Comparison between granulations	———			———	5% level
	Displacement Across Axis (D_l)				
Granulations	100	35	8	50	
Means of displacement	0.1121	0.0617	0.0318	−0.0957	
Level of significance of displacement	0.1%	5%	Not	0.1%	
Comparison between granulations	———	———		———	0.1% level
	———		———		5% level

[a] Any two means not underscored by the same line are significantly different while those underscored by the same line do not differ significantly.

prepared with granulation fraction 35 exhibit the best overall results.

A statistical evaluation of the difference between the absolute mean values of core tilt "across" and "along axis" of die table movement for each tablet press indicated no significant difference of tilt for "along" or "across axis."

Since the results obtained for tilt and displacement of core in the tablets produced on the Manesty and Kilian were so different, it was unreasonable to attempt a pooled analysis of the data for both machines.

GENERAL DISCUSSION

From the data presented under horizontal and

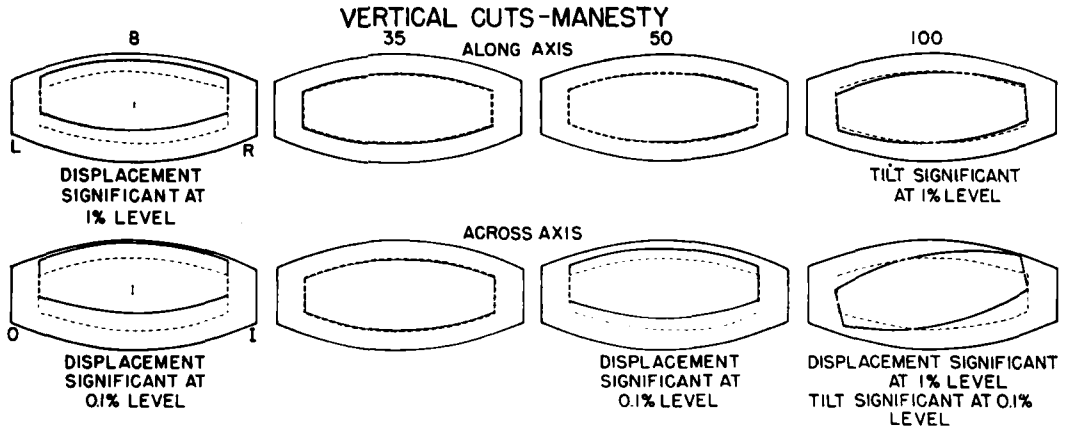


Fig. 11.—A graphic illustration indicating the influence of coating granulation size on core tilt and displacement "along" and "across" axis of die table movement for vertical cuts of tablets produced on the Manesty machine.

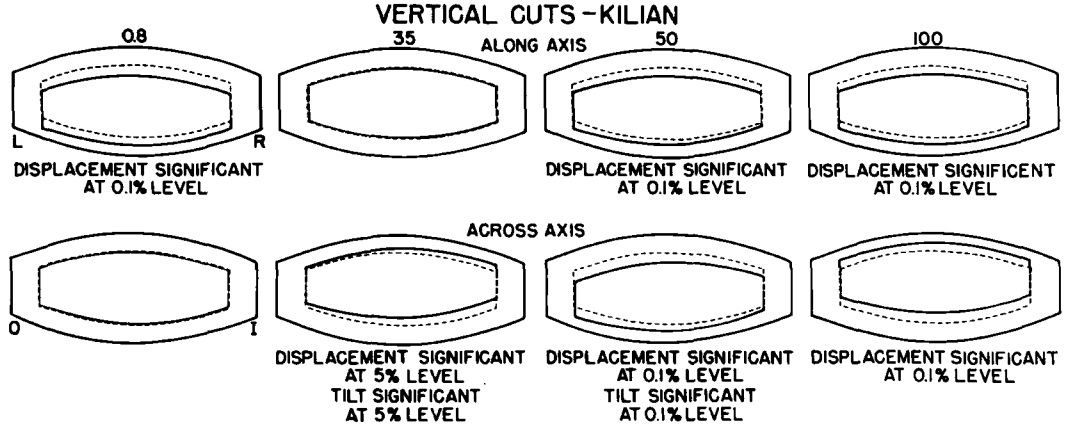


Fig. 12.—A graphic illustration indicating the influence of coating granulation size on core tilt and displacement "along" and "across" axis of die table movements for vertical cuts of tablets produced on the Kilian machine.

vertical core off-centering, it became evident that the cause for core dislocation in the horizontal cuts and core tilting and displacement in the vertical cuts could be explained by certain basic laws of physics. It seems that there are two major forces which are responsible for core dislocation, and these will be discussed in the subsequent paragraphs of this section.

As a result of the die table rotation, two forces are exerted on the tablets, one being centrifugal and the other tangential. Since the density of the core is greater than that of the coating granulation in the die, these two forces will tend to displace the core in the coating bed before the final compression step in the formation of the compressed coated tablets. The centrifugal force will tend to displace the core in the radial direction while the other force, which is tangential to the movement of the die table, will cause core displacement in the direction opposite to the rotation of die table. This effect is graphically illustrated in Fig. 13. The tangential force is dependent upon the direction of die table movement, while the centrifugal force is in the same direction for both machines. This effect is illustrated in Fig. 10, where the core is dislocated to the right and outside for the Manesty and toward the left and outside for the Kilian machine.

In the vertical plane, "across the axis" of die table movement, centrifugal force is active in causing the core to tilt toward the more stable vertical position. Because of the constraining properties of the coating granulation bed on the core, the only possible movement toward the vertical position is the lifting of the inside edge of the core in the upward direction. The core is also displaced in the upward direction along the vertical axis of the tablet. This is illustrated in Fig. 11.

For the vertical "along axis" core centration measurements, the tablets produced on the Manesty machine showed lifting of the right edge of the core and for those produced on the Kilian machine lifting of the left edge. This tilting of the core can be caused if the bottom bed of coating granulation onto which the core is deposited lies at a slant, instead of in a flat position in the die. The rotational movement of the die table would be expected to cause the bottom bed of coating granulation in the die to accumulate to a larger amount at the rear

edge of the die wall which is on the right for the Manesty and on the left for the Kilian. This larger amount of coating granulation at edge of the die would cause the coating bed to take on a tilted position in the die cavity. Subsequently, when the core tablet is deposited, it will take the tilted position of the coating bed. In the Kilian machine, where the core is imbedded into the coating bed by the free fall of the upper punch before the upper layer of coating granulation is added, the "along axis" tilt would be expected to be less due to the overall flattening out of the coating bed by the free fall of the upper punch. This was found to be the case and can be seen from the drawings in Figs. 11 and 12.

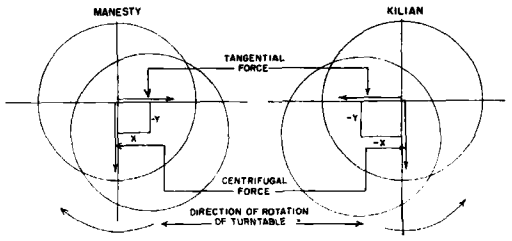


Fig. 13.—A graphic illustration showing the direction of tangential and centrifugal force acting to displace core along horizontal plane for Manesty and Kilian machines.

It is apparent from the vertical cut data in Table VII that the displacement of core along the vertical axis takes place in the upward direction for tablets prepared on the Manesty machine. In this tablet press, the core is transferred to the bottom bed of coating granulation by a transfer arm and deposited into the coating bed by a transfer pin, causing the tablet to fall into the bed center. Coating granulation is then added and the upper punch comes down to compress the tablet. Here it would be expected that the displacement of the core would be in the upward direction along the vertical plane.

For the tablets produced on the Kilian machine, the core shows a downward displacement when measured "along axis" and an upward displacement when measured "across axis" as shown by the data in Table IX. The vertical core displacement in the Kilian machine may be caused by the free fall action of the upper punch to imbed the core in the bottom bed of coating granulation before the upper layer of coating granulation is added. Since there is core displacement in both directions for this machine, the responsible factors are rather complex and will not be considered at this time.

As is apparent by comparing the data for the horizontal cuts, Tables III and IV with those of the vertical cuts, Tables VI to IX, the effect of the centrifugal and tangential forces are more pronounced on the dislocations of core on the horizontal cuts than the tilts of core on the vertical cuts.

From the data obtained for core dislocation along horizontal cuts and tilt and displacement along vertical cuts, there seems to be a definite relationship between degree of core off-centering and the size distribution of coating granulation. In addition, the machine design (Kilian and Manesty) appears to influence this effect.

On the basis of all the data for the Kilian and Manesty machines it is evident that coating granulations 100 and 50 yield tablets exhibiting the poorest core centration for the Manesty and Kilian machines, respectively. On the other hand, it appears that optimal core centration is obtained with coating granulation fraction 35 for the Manesty machine and with coating granulation fractions 8 or 100 for the Kilian machine. This relationship also holds when only the data from horizontal cuts are compared. However, a comparison of the data from the vertical cuts shows that for both the Manesty and Kilian machines, coating granulation fraction 35 gives the smallest displacement of core and coating granulation fraction 8 gives the least tilt of core. It becomes evident from these off-centering data that the dislocations of core from the horizontally determined cuts exert the predominant effect on the overall centration of core in tablet.

From the core expansion data reported in Table V, it is evident that the core expansion is smallest when coating granulation 100 is used. This has been found to hold for tablets produced on both the Manesty and Kilian machines. Since the sieve analysis on granulation 100 has shown it to have the smallest size distribution granules of the four coating granulations studied, it would be expected to cause the least core expansion. This may be explained on the basis that the smaller the particles, the less void space there is between particles and consequently, less space for the core to expand during the final compression step in the bed of coating granulation.

SUMMARY

The information obtained from this investigation may be summarized as follows:

1. A procedure has been developed for the determination of core dislocation in compressed coated tablets.

2. These factors appear to be responsible for core dislocation: (*a*) Two major forces which are acting tangentially and radially. (*b*) The bottom bed of coating granulation taking on a slanted shape due to die table rotation.

3. The data obtained in this study show that the core dislocation measured along the horizontal cuts have a more pronounced effect on the overall off-centering than the core tilt measured along the vertical cuts.

4. The results obtained indicate that the coating granulation size distribution influences core centration. In addition, this influence is not the same for the Manesty and Kilian machines.

5. Core expansion was observed to take place for each coating granulation, the least being with the coating granulation containing the smallest granules.

6. Using this procedure for determining core dislocation it should be readily possible to determine the optimal granule size distribution for a

particular coating granulation required to give optimal core centration.

7. Optimal core centration is desired for several reasons, the major ones being (*a*) Uniformity of dose, *i.e.*, if the tablets were to be broken along a center bisection, unequal doses would be obtained if the core is not in the center of the tablet. (*b*) Protective action of coating, *i.e.*, if the coating is used to improve the stability of the core ingredients, conceal the bitter taste of medicaments used in the core, and the like, the coating must completely cover the core tablet, otherwise the effectiveness of the coating is reduced. (*c*) Physical appearance, *i.e.*, if the core was to protrude out of the coating, either due to tilting or displacement in the vertical plane or horizontal dislocation, it would give an unsatisfactory aesthetic appearance.

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Study of the Thermistor Bridge for the Measurement of Colligative Properties

By FRANK M. GOYAN and RICHARD D. JOHNSON

Further refinement of the apparatus described by Goyan and Reck has been studied, using thermistors. One variation described involves the use of intermittent power with simultaneous recording. Another variation treats the problem of using relatively high battery voltages in order to increase the sensitivity of the bridge without the use of amplifiers. The paper discusses the determination of molecular weights, as well as osmometry and isotonicity measurements. Results indicate that significant measurements can be made without minimizing the heating produced by the bridge current.

THERMISTORS may be used as resistance thermometers with high negative temperature coefficients. Because of their small size and low cost as compared to platinum resistance thermometers and their high coefficient; they seem to be ideally suited to the determination of temperature changes associated with freezing-point lowering and boiling-point elevation. One of the authors (1) has described an isotonicity meter based upon freezing-point measurements which includes a single thermistor in a Wheatstone bridge. The off-balance condition of the bridge is read on a vacuum-tube voltmeter. Ballard and Goyan (2) have shown that isotonicity in terms of per cent sodium chloride is a linear function of the usual colligative properties within the range of concentration and accuracy normally required.

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It is quite proper to generalize that any osmometer, within the special limitations of each instrument, measures some approximately linear function of the colligative properties of solutions. The Hill-Baldes thermoelectric osmometer is no exception. In its original form it consisted of a thermocouple arranged in a constant temperature humidified air chamber (3). By placing a drop of solvent on one junction of the thermocouple and a drop of solution on the other, a temperature increase of the drop of solution relative to the drop of solvent develops measurable thermal E.M.F. This temperature difference and the resulting E.M.F. is proportional to the isotonicity or osmolal concentration of the solution.

With the advent of thermistors, many investigators (4–6) have modernized the Hill-Baldes osmometer by placing two thermistors in the chamber and connecting them as two arms of a Wheatstone bridge. This change has the advantage that the electric circuits are easier to

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With the advent of thermistors, many investigators (4–6) have modernized the Hill-Baldes osmometer by placing two thermistors in the chamber and connecting them as two arms of a Wheatstone bridge. This change has the advantage that the electric circuits are easier to

deal with because of the higher voltage generated per degree of difference in temperature. However, the disadvantage of thermistors over thermocouples is that the former are heated by the current needed to operate them. This problem has been mentioned by most investigators. Their assumption that the heating effect may safely be neglected seems to be justified by their results. However, the present study has shown that good results can be obtained when the heating produced by the measuring current is greater than that produced by the phenomenon itself.

Goyan and Reck (7) made a radical modification of the Hill-Baldes osmometer by placing the two junctions of their thermocouple in separate tubes, each lined with platinum gauze for the purpose of holding solvent as close as possible to the junctions. This method gave good results and suggested the idea that the method is essentially a modification of the boiling-point method for determining colligative properties. Higuchi, *et al.* (8), followed this idea to its logical conclusion by evacuating the chamber after placing their solvent and solution in contact with thermistors. By means of mechanical stirring, they were able to simulate boiling-point work at room temperature and show that their results could be treated by classical thermodynamics.

When the need for accurate molecular weight determinations came to the attention of one of the authors, it was decided to modernize the Goyan-Reck apparatus by using thermistors. The present paper deals with this phase of molecular weight studies. It is obvious that any adequate measure of colligative properties will give a real or (in the case of dissociating or associating solutes) an apparent molal concentration. Since the weight of solute is known for some given quantity of solvent and the number of moles of solute in the same quantity of solvent is easily read from a suitable calibration chart of the instrument, it follows that the Goyan-Reck apparatus should be as effective in determining molecular weight as in determining isotonicity values.

This research began in an exploratory way. The first phase represents various attempts to minimize heating effects due to current through the thermistors. The second phase is a deliberate attempt to operate the thermistors at high current in order to take advantage of the greater sensitivity at the higher bridge voltages.

EXPERIMENTAL

Figure 1 shows the final modification of one of the sample cells using thermistors. During the first phase of this investigation, the tubes were 9.5 cm. long. Both were slipped through holes in a rubber stopper into water in a wide-mouth, 400-ml. Dewar filler having an internal diameter of 4.5 cm. The water in the Dewar flask was stirred vigorously with a stirring motor and a screw or propeller at the end of a free shaft. In the early part of the investigation it was thought desirable to adjust the temperature by changing the rate of stirring, but later a small coil of resistance wire (about 10 ohms) was wound inside a glass tube to serve as a heater. The heating was controlled manually by means of a variable transformer connected to a low-voltage transformer. When manual control of temperature became arduous, a thermocap relay was arranged to short an adjustable resistor in series with the heater. It was found that the value of this resistor should be such that the change in temperature was extremely gradual. Under these conditions, temperature corrections could be made for variations of 0.01–0.02°. Temperature was read on an 18–28° thermometer graduated in 0.01° marks. The same thermometer also served to operate the thermocap relay.

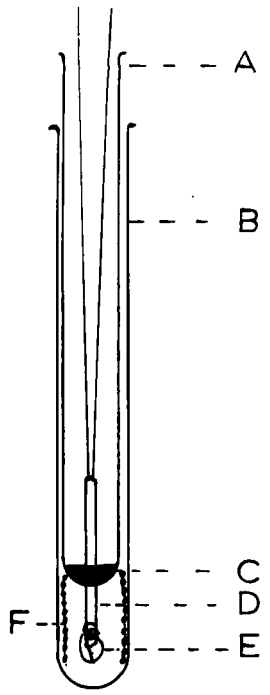

Fig. 1.—Thermistor assembly used in a constant temperature water bath maintained at 25° (about 5° above room temperature). A = glass tube supporting thermistor; B = glass tube selected so the thermistor support barely clears (7.5 mm. x 12 cm. I.D.); C = dental amalgam holding thermistor in place; D = thermistor as supplied at the end of a glass rod; E = platinum loop; F = platinum gauze.

The electrical circuit for the first phase of the investigation has been described previously (9). The thermistor[1] used for the sample had a temperature coefficient of −4.8% per degree and a resistance

[1] The thermistors were type 51A1 from the Victory Engineering Corp. of Union, N. J., although not specially selected.

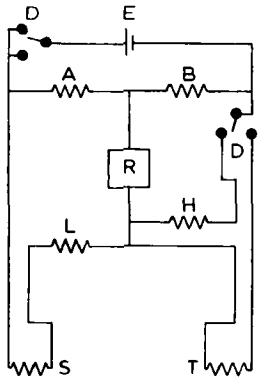

Fig. 2.—Circuit diagram of the bridge used for the high voltage work. A and B = ratio arm box; D = D.P.D.T. switch; E = battery (see text); H = Heathkit decade resistance box, 0–99,999 ohms; L = Leeds and Northrup box (see text); R = Leeds and Northrup Speedomax recording potentiometer 0–10 mv.; S = thermistor used for sample; T = thermistor used for reference.

of about 94,000 ohms at 25°. The ratio-arm box was used at the 1,000 settings. The switch built into the recorder for making and breaking external circuits was described in connection with incremental titration (9). This switch was used to open and close the battery circuit of the bridge at regular intervals, the closed condition lasting for a few seconds and the open condition about half a minute. Each time the battery circuit was closed the recorder would draw a horizontal line, the length of which was found to be a measure of the off-balance condition of the bridge. Consequently, the length of the lines for various known concentrations could be plotted against concentration in order to obtain a calibration chart.

Samples were added with a medicine dropper to the platinum loops surrounding the thermistor after removing the tube from the bath. A small piece of sheet brass was bent to form a channel to assist in removing the thermistors from the tubes without touching the sides. A slot was cut for the lip of the tube and the upper half above the slot built up with tape so that the surface of the tape was flush with the inner surface of the tube. The thermistor, after washing and filling, was replaced in the tube and the tube replaced in the water bath. During this operation the thermistors were connected but the battery circuit was open.

Figure 2 shows a circuit diagram of the bridge used in the second phase of the work. The positions of the battery and of the recorder were exchanged in developing this circuit from the one previously published (9). This was done in order to gain sufficient sensitivity without the amplifier. During a few experiments, a 10,000-ohm resistor was added to each ratio arm, but most of the work was done with the same 1,000-ohm ratio-arm resistors used previously.

It must not be assumed that the experimental details given above represent the authors' recommendation for a finished instrument. The object of the work was to study the design problem and to report significant information. For example, the switching arrangement designed to substitute a resistance box for the thermistor used as a reference resistor was a great convenience in measuring absolute temperature changes, but did not contribute to the functioning of the instrument as an osmometer.

All solutions used were prepared by weighing chemicals from good quality commercial samples on an analytical balance. All of the solutions were made with distilled water as the solvent and distilled water was always used on the reference thermistor.

RESULTS

Phase I

The first phase of the work showed that thermistors could be substituted for thermocouples in the Goyan-Reck apparatus without reducing sensitivity. The advantage of this substitution is that there is no need to take great pains to avoid parasitic thermal potential differences, thus eliminating the critical need for a constant-temperature air bath. It was further established that when the current from a $1^1/_2$-v. battery passed through the bridge for a few seconds at half-minute intervals, the line drawn by the recorder pen varied in length as a function of the off-balance condition of the bridge. Within limits, the length of these lines is a linear function of the concentration of solution on the sample thermistor. Figure 3 shows a typical recording and Fig. 4 shows the accumulated results of several measurements.

The feasibility of this intermittent method is interesting, but it failed to produce a dramatic improvement in results over what might be expected from a thermocouple and a good amplifier. It was discovered that the limit of accuracy was determined by the geometry of the drop with respect to the platinum gauze, the design of the water bath, rate of stirring, and freedom from rapid temperature changes in the bath. One important

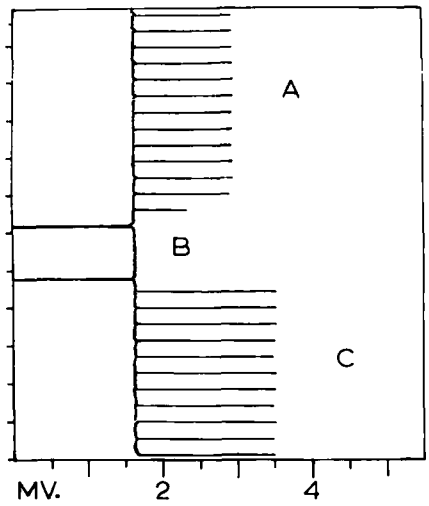

Fig. 3.—A typical recording using the intermittent method at $1^1/_2$ v. on the bridge. The graph shows millivolt values at regular time intervals. A = water; B = open circuit; C = 0.0435 *M* dextrose. Each division on the ordinate represents approximately 100 sec.

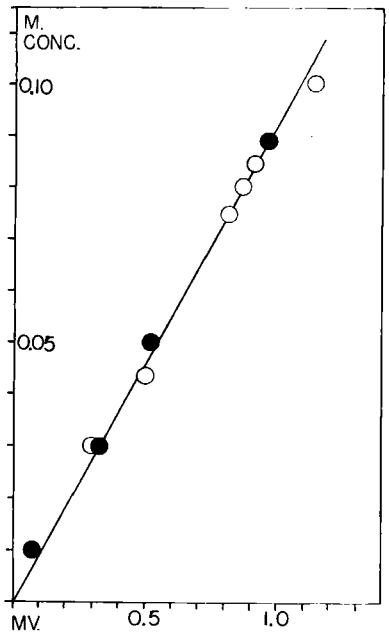

Fig. 4.—Calibration chart for the intermittent method: ●, sucrose; ○, dextrose. (Molar concentration *vs.* difference between mv. reading for water and solution.)

consideraton in the design of the water bath is that there should be an air space between the rubber stopper and the surface of the water, thus indicating that the use of a Dewar flask is important.

Phase II

The second phase of this study was suggested by the results of the first. Substitution of thermistors in place of thermocouples made it possible to simplify the electrical circuit. It seemed reasonable to explore the use of higher battery voltages across the bridge so as to obtain greater off-balance voltage for the same change in thermistor resistance. It is easy to show that, to a good approximation, for a balanced bridge of the type shown in Fig. 2

$$\Delta E = \Delta R(E/4R)$$

ΔE is the potential difference seen by the recorder; E is the battery voltage; R is the resistance of either thermistor arm; and ΔR is a small change in R. This equation was verified in practice. One-hundred ohms variation of the box in series with the sample thermistor showed 1.4 mv. for a 6-v. battery, 2.9 mv. for a 12-v. battery, and 5.9 mv. for a 24-v. battery.

The easy conversion from millivolt reading to ohms is a great help in reading the bridge. It is not necessary to balance except to the nearest 100-ohm setting. Two positions on the recorder chart separated by the voltage corresponding to 100 ohms are located to the right and left of the nul position by approximately balancing the bridge. The exact resistance required to balance is then easily calculated.

The use of the recording potentiometer as a nul instrument for a Wheatstone bridge would seem needlessly complex except for the fact that the equation given above is valid only when very little current is flowing through the nul instrument. Another and more essential use of the recorder is to establish final equilibrium values. Figure 5 shows a typical example of a slow and a moderately rapid rate of attainment of equilibrium after the sample had been exposed to the air of the room. Solutions seem to come to a steady state more slowly than pure water, indicating that in some cases diffusion of a film of condensed water is a problem when the drop was initially cooled by evaporation. The general behavior of the samples indicates the desirability of making a hole close to and parallel to the thermistor through the glass tubing support in order to insert a long hollow needle for washing and filling without removing the thermistor from the bath. This is an excellent feature of a commercial instrument (10). An innovation which seems to hasten equilibrium is to wait a few minutes before connecting the battery to the bridge. Convection caused by the heating of the thermistor probably provides some convection stirring which differs under different conditions.

The method used for placing the sample has some advantages, however. It is simple and makes it easy to wash and dry the thermistor before adding a sample, thus reducing the volume of solution required to about 0.1 ml. For exploratory work of the type reported, there is considerable advantage in frequent inspection. Several experiments with solutions of potassium permanganate showed that one of the platinum loops could be washed with 10–20 drops of new solution.

The most important conclusion that can be drawn from this work is shown in Fig. 6 and Table

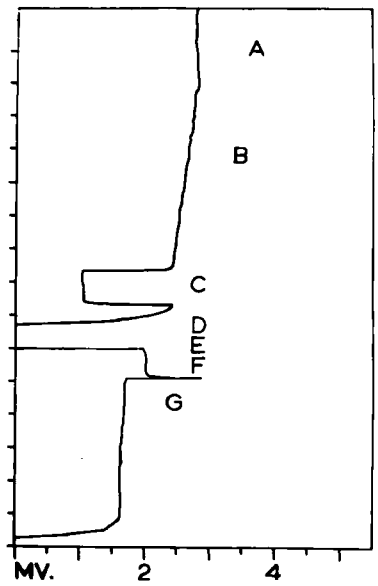

Fig. 5.—A typical recording using 6 v. across the bridge. (Time *vs.* mv.) A = final value for 0.1 *M* urea; B = slow attainment of steady state; *C* = change of decade box by 100-ohms; D = rapid approach to approximate equilibrium; E = thermistor first connected; F = battery circuit open; G = final equilibrium value for water. Each division on the ordinate represents approximately 100 sec.

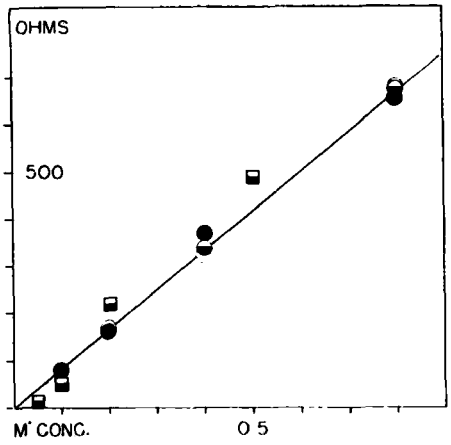

Fig. 6.—Cumulated results at several different battery voltages. Open figures = 6 v., half-open figures represent 12 v. and dark figures represent 24 v. Circles represent urea; and squares, sucrose. Molar concentration is plotted against the difference between water and solution in ohms

I. It will be noted that high voltages across the bridge can be used without evidence of serious trends. The temperatures shown in Table I were

TABLE I.—A STUDY OF AQUEOUS SOLUTIONS OF UREA AT DIFFERENT BATTERY VOLTAGES[a]

	Resistance Change from Value with Water, ohms		
M	6 v.	12 v.	24 v.
0.1	72	69	82
0.2	159	167	160
0.4	319	337	371
0.8	685	676	650

[a] Temperature elevation due to current: 0.07° at 6 v., 0.20° at 12 v., 0.63° at 24 v.

calculated from data taken with the bath at 25° and the resistance box substituted for the standard thermistor. Water was placed on the sample thermistor and readings taken at different bridge voltages. The first reading at $1^1/_2$ v. was taken as the true resistance of the thermistor at 25°. As the voltage on the bridge was increased, the resistance went down. The differences were converted to temperature from the measured temperature coefficient and resistance of the thermistor.

Although there seems to be no evidence that the results obtained with high voltage across the bridge are invalid, there are some anomalous results that may indicate that the steady states developed on the thermistor are influenced by other factors. This has been recognized for some time (3), and it is possible that the excessively warm drops amplify this effect. Figure 6 displays the results obtained with sucrose. It will be noted that the points for this substance deviate from a straight line by an amount greater than can be attributed to experimental error.

DISCUSSION

This work is of value in the development of improved instrumentation based upon the Hill-Baldes principle, although the authors are not presenting a finished instrument. It is of special interest that high voltages can be used successfully even though the resulting heating of the thermistor amounts to a change of resistance of almost 3,000 ohms or nearly three-quarters of a degree.

Neither method was pushed to its fullest extent either with respect to accuracy or convenience. However, a study of the data used to plot Fig. 4 shows an average deviation of 1.5% for the three points above 0.01 *M*. All of the dextrose points show an average deviation in the calculated slope of a hypothetical straight line of 3.6%. It is safe to conclude that routine measurements could be made with less than 3% error for the intermittent method.

No attempt was made to refine the techniques used in obtaining data for Table I. Most of the values reported were obtained from duplicate determinations. The maximum difference was 14%, the minimum difference was 0.3%, and the average difference was 5%. This was sufficient to show a lack of significant trend as a function of battery voltage and indicated that the use of higher current might be expected to give satisfactory molecular weights. A systematic determination of the molecular weight of an unknown compound using a 6-v. supply showed an average deviation of 1.8% for nine readings. It is not to be assumed that this value necessarily represents the ultimate accuracy obtainable.

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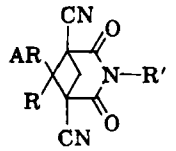
Chemistry of Guareschi Imides II. Enolic Character of β-Aryl Guareschi Imides

By ARNOLD A. LIEBMAN† and FRANK E. DIGANGI

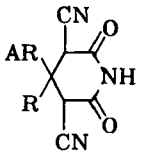
The bicyclization and methylation reactions of β-aryl Guareschi-like imides were studied and the results indicate a favoring enolic structure that hindered these reactions in alkaline media.

With the successful lithium aluminum hydride reduction of β,β-dialkyl-bicyclo Guareschi imides I (1), it appeared that a series of β-alkyl-β-aryl-bicyclo Guareschi-like imides II might also be prepared which upon reduction would yield compounds having a potential analgesic structure III.

I R, R′-alkyl
R″-H, -CH_3



II AR-C_6H_5 R-alkyl
R′-H, -CH_3



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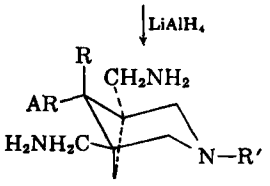
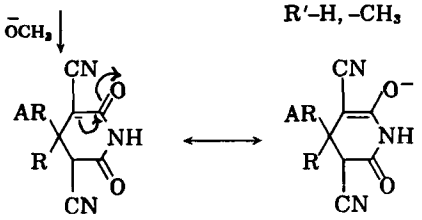


III AR-C_6H_5 R-alkyl
R′-H, -CH_3



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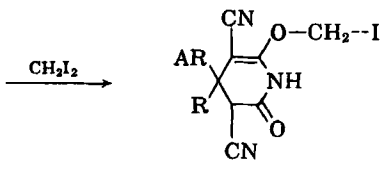

VI

Since the Guareschi reaction is limited to ketones having two methyl or methylene groups adjacent to the carbonyl, starting materials were prepared *via* the two step synthesis developed by Cope (2) for the preparation of ethyl-(1-arylalkylidene)-cyanoacetates followed by the Michael addition of sodium cyanoacetamide using McElvain's (3) procedure.

Treating the β-aryl Guareschi-like imides with sodium methoxide and methylene iodide in the same manner as the β,β-dialkyl Guareschi imides (1) resulted in the recovery of starting material only. Similar results were encountered when α,α′-dicyano-β-methyl-β-phenylglutarimide was converted into its sodio derivative and treated with 1,2-dibromoethane and 1,3-diiodopropane, respectively. The β-ethyl analog also failed to form a bicyclo product with methylene iodide in sodium methoxide solution.

Failure of these β-alkyl-β-aryl Guareschi-like imides IV to form bicyclo heptanes in a manner similar to the β,β-dialkyl Guareschi imides may be attributed to either steric hindrance or an enolic structure inhibiting the reaction. The former may be eliminated since, α,α′-dicyano-β-isobutyl-β-methyl-glutarimide has been synthesized and it readily underwent the expected bicyclization with methylene iodide (1). On the other hand, dissolving a β-aryl Guareschi-like imide IV in alkoxide solution could result in formation of the enolate anion V which could react with methylene iodide yielding the enol ether system VI. This would decompose to starting material when treated with aqueous acid.

Methylation of α,α′-dicyano-β-methyl-β-penyl-glutarimide indicated the enolic nature of this compound. When this imide was converted into its mono sodium salt and treated with one molecular equivalent of methyl iodide, reaction appeared to occur since the solution quickly became neutral to moist litmus paper. However, only starting material was isolated from the reaction mixture. When two equivalents of sodium ethoxide and two of methyl iodide were used, a monomethyl product resulted. The entering methyl group attached itself to the imide nitrogen as evidenced by the absence of N—H stretching bands in the infrared spectrum of this compound. A dimethyl compound was obtained when this imide was treated with three equivalents of sodium ethoxide and of methyl iodide. The dimethyl compound was also obtained when six equivalents of sodium ethoxide and of methyl iodide

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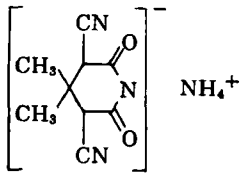
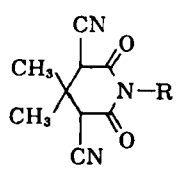
were used. These results are for the most part consistent with those of Kon and Thorpe (4) obtained in methylation studies of β,β-dialkyl Guareschi imides.

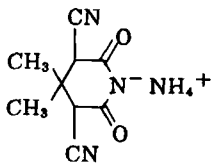
The enolic nature of Guareschi imides was further demonstrated by methylation of the ammonium salts of the imides obtained from the Guareschi reaction. Suspending the ammonium salt of α,α′-dicyano-β,β-dimethylglutarimide (VII) in an ethanol-



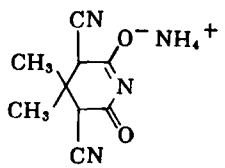
VII



VIII R-H
IX R-CH_3



X



XI

benzene mixture and adding a slight excess of dimethyl sulfate resulted in a clear solution which was subsequently diluted with water. Upon standing, the solution precipitated a mixture of products, apparently the free imide VIII and the N-methylimide IX. Significantly, the precipitated material consisted entirely of neither VIII nor of IX, indicating a starting mixture of amonium salts X, XI. Similar results were encountered when the ammonium salt of α,α′-dicyano-β,β-pentamethyleneglutarimide was treated with dimethyl sulfate.

EXPERIMENTAL[1]

Ethyl-(1-phenylethylidene)-cyanoacetate.—In a 1-L., three necked, round-bottom flask equipped with a stirrer and Dean-Stark water separator were placed 120 Gm. (1 mole) of acetophenone, 113 Gm. (1 mole) of ethyl cyanoacetate, 48 Gm. (0.8 mole) of glacial acetic acid, 15.4 Gm. (0.2 mole) of ammonium acetate, and 200 ml. of anhydrous benzene. The mixture was treated according to the procedure of Cope (2) and McElvain (3) and upon distillation yielded 117 Gm. (54%) of product boiling between 145–160°/0.7 mm. Reported (3) boiling range is 135–160°/0.35 mm.

α,α′ - Dicyano - β - methyl - β - phenylglutarimide.—According to the procedure of McElvain and Clemens (5), 0.5 mole of sodium cyanoacetamide was treated with 0.5 mole of ethyl-(1-phenylethylidene)-cyanoacetate. The dried product weighed 114.4 Gm. (90%) and melted at 272–278° (decompn). Reported (5) melting point is 274–278° (decompn).

Attempted Preparation of β-Alkyl-β-aryl-bicyclo Guareschi Imides.—In a 1-L., round-bottom flask fitted with a reflux condenser were placed 275 ml. of absolute methanol and 17.2 Gm. (0.75 Gm. atom) of sodium, added in small portions. When the sodium had dissolved, 63.3 Gm. (0.25 mole) of α,α′-dicyano-β-methyl-β-phenylglutarimide was added and the mixture heated under reflux for ½ hour during which time the imide dissolved. To the solution, 101.0 Gm. (0.38 mole) of methylene iodide was added and reflux continued for 3 hours during which time the methylene iodide dissolved. The solution was cooled and poured with stirring into 325 ml. of water containing 72 ml. of concentrated hydrochloric acid. When precipitation was complete, the solid was removed and washed repeatedly with ether. Recrystallization of this material from methanol and acetone yielded 39.1 Gm. of starting material. This experiment was repeated under extremely anhydrous conditions, using highly purified starting material and reagents. The solid that was obtained was recrystallized from absolute ethanol and melted at 282°. A mixed melting point with starting material caused no depression and the infrared spectrum was identical with that of starting material. Similar results were obtained when these β-aryl Guareschi-like imides were treated with 1,2-dibromoethane and 1,3-diiodopropane. In the latter instance, the above conditions were observed and in addition, the reaction was attempted in tetrahydrofuran using sodium hydride as the base.

Reaction of α,α′-Dicyano-β-methyl-β-phenylglutarimide with One Molecular Equivalent of Methyl Iodide.—A solution of sodium ethoxide was prepared by adding 0.92 Gm. (0.04 Gm. atom) of sodium to 50 ml. of absolute ethanol. To the solution, 20.1 Gm. (0.04 mole) of α,α′-dicyano-β-methyl-β-phenylglutarimide suspended in 200 ml. of absolute ethanol was added with stirring. The reaction mixture quickly became homogeneous and 6.0 Gm. (0.044 mole) of methyl iodide was added with stirring over a period of 10 minutes. The mixture was stirred and heated under reflux for 15 minutes when a sample was neutral to moist litmus paper. Reflux was continued for 1 hour after which time 150 ml. of ethanol was removed by distillation under reduced pressure. The remaining solution was cooled and poured into 300 ml. of water containing 25 ml. of concentrated hydrochloric acid. The precipitated material was collected, washed with ether and recrystallized from ethanol, melting at 282–285° (decompn). A mixed melting point with starting material gave no depression in the melting point. The infrared spectrum of this material was identical to that of starting material.

α,α′ - Dicyano - β - methyl - β - phenyl - N - methylglutarimide.—The conditions in the preceding experiment were followed using 12.7 Gm. (0.05 mole) of the imide, 2.3 Gm. (0.1 Gm. atom) of sodium and 15.6 Gm. (0.11 mole) of methyl iodide. The reaction mixture was neutral to moist litmus paper when the methyl iodide had all been added. The neutral solution was refluxed for an additional hour when it was evaporated under reduced pressure to a volume of 100 ml. and diluted with 300 ml. of water followed by 25 ml. of concentrated hydrochloric acid. An oil separated which was solidified with stirring and recrystallized from ethanol yielding 6.6 Gm. (49%) of the monomethyl product melting between 172–181°. The infrared spectrum of this compound differed with that of starting material in that no N—H bands were present.

[1] Melting points are uncorrected and were taken with a Thomas Hoover capillary tube melting point apparatus. Microanalyses were performed by the Microanalytical Laboratory of the University of Minnesota.

Anal.—Calcd. for $C_{15}H_{13}N_3O_2$: C, 67.40; H, 4.90. Found: C, 67.47; H, 5.04.

α,α' - Dicyano - α' - β - dimethyl - β - phenyl - N-methylglutarimide.—Using the above procedure, but using 0.15 Gm. atom of sodium and 0.165 mole of methyl iodide to 0.05 mole of the imide, yielded 10 Gm. (71%) of the product, m.p. 190–192°.

Anal.—Calcd. for $C_{16}H_{15}N_3O_2$: C, 68.32; H, 5.38. Found: C, 68.29; H, 5.37.

Similar results were obtained with a 2:1 ratio of methyl iodide to each active hydrogen and the solution refluxed for 21 hours. Assignment of the second entering methyl group to the α'-position rather than the α is an arbitrary one.

Reaction of the Ammonium Salt of α,α'-Dicyano-β,β-dimethylglutarimide with Dimethyl Sulfate.—The ammonium salt of α,α'-dicyano-β,β-dimethylglutarimide, 52.0 Gm. (0.25 mole), was suspended in a mixture of 100 ml. of anhydrous benzene and 100 ml. of absolute ethanol contained in a 500 ml., three necked, round-bottom flask equipped with a stirrer, dropping funnel, and reflux condenser. The mixture was stirred and heated under reflux for 1 hour after which time 37.8 Gm. (0.3 mole) of dimethyl sulfate was added with stirring. After the addition was completed, the mixture was stirred and heated under reflux for 2 hours. Within the first 15 minutes of this reflux period, the solid had all dissolved. The solution was cooled, diluted with an equal volume of water and refrigerated overnight. The solid that had separated (31.8 Gm.), was collected, and recrystallized from 60% ethanol. This material melted between 139–155°. A mixed melting point of α,α'-dicyano-β,β-dimethylglutarimide and α,α'-dicyano-β,β-dimethyl-N-methylglutarimide melted at 144–155°.

SUMMARY

1. β-Aryl Guareschi-like imides failed to undergo bicyclization with α,Ω-dihalides.

2. The failure of β-aryl Guareschi-like imides to undergo bicyclization is attributed to a favored enolic system in the presence of alkali which in turn resists the expected reaction.

REFERENCES

(1) Liebman, A. A., and DiGangi, F. E., THIS JOURNAL, **52**, 276(1963).
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(3) McElvain, S. M., and Clemens, D. H., *ibid.*, **80**, 3915 (1958).
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Mercury(II) Complex of *d*-Cycloserine

By NICHOLAS G. LORDI

The apparent formation constant of the 2:1 complex of *d*-cycloserine and mercury (II) has been measured and found to be $10^{17.5}$ at 25° in solutions of ionic strength of ~0.4. Evidence is presented to substantiate the conclusion that chelation contributes little to the structure of the complex.

As PART of a study dealing with the influence of metal ions on the mode of degradation of the antibiotic *d*-cycloserine (*d*-4-amino-3-isoxazolidone) in aqueous solution, the apparent formation constant of the mercury(II) complex of cycloserine was measured. Neilands (1) and Neuzil and Breton (2) have indicated that cycloserine formed 1:1 and 2:1 complexes with copper(II), zinc(II), cadmium(II), cobalt(II), and nickel(II). No significant complex formation was observed with beryllium(II), magnesium(II), manganese(II), iron(II), and iron(III). It was of interest to compare the stability of the cycloserine–mercury(II) complex with the stabilities reported for similar complexes with other metal ions, as well as with several of the metal ion complexes of other ligands.

EXPERIMENTAL

A potentiometric technique was employed (3, 4). The potential of a mercury electrode in equilibrium with a solution of the mercury(II)–cycloserine complex in the presence of excess cycloserine was measured as a function of pH. Solutions were prepared containing from 0.01 to 0.05 *M* cycloserine, equimolar amounts of sodium hydroxide, from 0.0004 to 0.001 *M* mercuric acetate,[1] and sufficient potassium nitrate to adjust their ionic strength to 0.4. The potential of the mercury electrode (Sargent No. S-30438) *vs.* a saturated calomel electrode (S.C.E.) isolated from the solution through a 1.5 *M* potassium nitrate agar salt bridge was measured using a Fisher student potentiometer. The pH of the solution was varied by titration under nitrogen with 1 *M* perchloric acid. The resulting volume changes amounted to less than 8 per cent of the initial volume at the end of the titration. Corrections were made for concentration changes owing to dilution when significant. A Beckman model G pH meter was employed, using an external glass electrode (type 1190-80) to measure the pH. Since the complex was observed to precipitate at pH values greater than 8, the potential measurements were restricted to the pH range 2–8. The temperature of the solution and calomel electrode was maintained at 25 ± 0.5°.

The apparent dissociation constants of the cycloserine were also measured in 0.4 *M* potassium

Received June 19, 1962, from the College of Pharmacy, Rutgers-The State University, Newark, N. J.
Accepted for publication July 10, 1962.

[1] The acetate and nitrate ions present in the system would have a negligible effect (other than that due to alteration in ionic strength) on the measured potential, owing to the much greater concentration of cycloserine than acetate and the very weak tendency of mercury(II) to complex nitrate.

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nitrate, by titration with sodium hydroxide and perchloric acid.

The cycloserine[2] had a melting point of 155° (decompn.) after recrystallization from alcohol. The other chemicals used were of reagent grade.

CALCULATIONS AND RESULTS

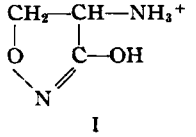
Table I summarizes the equilibria describing the state of a solution containing cycloserine and

TABLE I.—SUMMARY OF EQUILIBRIA AND APPARENT EQUILIBRIUM CONSTANTS DESCRIBING THE MERCURY(II)–CYCLOSERINE SYSTEM[a]

Equilibria	K′	Log K′
$H_2L^+ = HL + H^+$	K_{a1}	−4.57
$HL = L^- + H^+$	K_{a2}	−7.40
$Hg^{++} + HL = HgL^+ + H^+$	K_{10}	...
$Hg^{++} + HL = Hg(HL)^{++}$	K_{11}	...
$Hg^{++} + 2HL = HgL_2 + 2H^+$	K_{20}	2.73
$Hg^{++} + 2HL = HgL(HL)^+ + H^+$	K_{21}	8.67
$Hg^{++} + 2HL = Hg(HL)_2^{++}$	K_{22}	14.07
$Hg^{++} + HL + L^- = HgL(HL)^+$	$K_{23} = K_{21}/K_{a2}$	16.06
$Hg^{++} + 2L^- = HgL_2$	$\beta_2 = K_{20}/(K_{a2})^2$	17.53
$HgL(HL)^+ = HgL_2 + H^+$	$K_{31} = K_{20}/K_{21}$	−5.93
$Hg(HL)_2^{++} = HgL(HL)^+ + H^+$	$K_{32} = K_{21}/K_{22}$	−5.44

[a] In aqueous solution at 25°C. and $\mu \sim 0.4$.

mercury(II) and the equilibrium constants computed from the experimental data. In aqueous solution cycloserine may exist as a cation (H_2L^+), dipolar ion (HL), or anion (L^-), as indicated by structures I, II, and III. The observed values of K_{a1}' and K_{a2}', describing the equilibria between these forms, agreed with those reported by Neilands (1).



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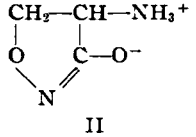

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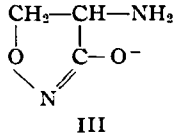


III

The experimental data were analyzed in a manner similar to that described by Brooks and Davidson (5). In order to simplify the calculations, it was assumed that the 1:1 complexes (equilibria 3 and 4) did not significantly contribute to the complexation of mercury(II) by cycloserine. This assumption was justified since measurements were made in the presence of excess ligand. It was substantiated by the subsequent analysis. K_{10} and K_{11} were not measurable under the conditions employed in this study.

[2] The cycloserine employed in this study was a sample of Seromycin, Lot No. 61, generously supplied by Eli Lilly & Co.

The total concentration of ligand species, therefore, is

$$\Sigma L = [L^-] + [HL] + [H_2L^+] + 2[HgL_2] + 2[HgL(HL)^+] + 2[Hg(HL)_2^{++}] \quad \text{(Eq. 1)}$$

while the total concentration of mercury(II) species is

$$\Sigma Hg(II) = (Hg^{++}) + [HgL_2] + [HgL(HL)^+] + [Hg(HL)_2^{++}] \quad \text{(Eq. 2)}$$

Since Σ Hg(II) $\gg$ (Hg^{++}), the concentration of uncomplexed ligand species, $[L^-] + [HL] + [H_2L^+]$, may be taken as $\Sigma L - 2\Sigma Hg(II)$. It is convenient to define the following function which may be derived from Eq. 2 and equilibria 5, 6, and 7

$$\frac{\frac{Hg(II)}{(Hg^{++})} - 1}{[HL]^2} = \frac{K_{20}}{(H^+)^2} + \frac{K_{21}}{(H^+)} + K_{22} = \bar{K} \quad \text{(Eq. 3)}$$

Also

$$[HL] = \frac{\Sigma L - 2\Sigma Hg(II)}{\alpha} \quad \text{(Eq. 4)}$$

where

$$\alpha = 1 + \frac{(H^+)}{K_{a1}} + \frac{K_{a2}}{(H^+)}$$

The mercuric ion activity was calculated from the measured potential of the mercury electrode *vs.* the S.C.E. using the Nernst equation (3)

$$E = 0.612 - 0.0296 \log (Hg^{++}) \quad \text{(Eq. 5)}$$

where 0.612 volts[3] is the standard potential of the Hg/Hg^{++} couple *vs.* the S.C.E. Corrections owing to the reduction of Hg^{++} by Hg^0 to Hg_2^{++} were negligible because of the excess ligand present. The mercury (I) complex of cycloserine is not stable, readily disproportionating.

Equation 3 was used to evaluate the constants K_{20}, K_{21}, and K_{22}. Values of the function $(\bar{K})$ between pH 6.5–8 were used to compute K_{20}; values between pH 4.5–6.5 were used to compute K_{21}; and values between pH 4.5–2 were used to compute K_{22}. The consistency of the experimental data with the proposed model is illustrated by Fig. 1 which shows a plot of log $\bar{K}$ as a function of pH. Values of the function on the left-hand side of Eq. 3 computed from the experimental data are compared to $\bar{K}$ calculated using the tabulated values of K_{20}, K_{21}, and K_{22}.

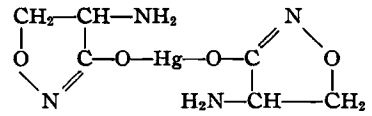
The apparent formation constant (β_2') and dissociation constants (K_{31}' and K_{32}') of the complex were calculated as indicated in Table I.

DISCUSSION

A comparison of the apparent formation constants reported for some metal ion (1, 6) complexes of *dl*-serine and *d*-cycloserine is made in Table II. As expected, the stability of the 2:1 cycloserine–mercury(II) complex is considerably greater than

[3] No attempt was made to correct the standard potential to a value corresponding to $\mu \sim 0.4$, because of a lack of information concerning the effect of ionic strength on the potential of the Hg/Hg^{++} couple. This meant that the value of the computed formation constant was high, probably by not more than 0.3 log K unit.

that observed for other metals. The known tendency of cycloserine to chelate copper(II), zinc(II), and cobalt(II) is a great deal less than that observed for serine. Neilands (1) has pointed out that this is probably due to the less favorably fixed positions of the potential chelating groups in cycloserine as contrasted to the flexibility of the amino acid molecule. On the other hand, the stabilities of the mercury(II) complexes are of the same order of magnitude. This observation, as well as the experimental data, supports the view that chelation does not significantly contribute to the structure of the complex, which may be represented at pH ~8 as



The dissociation constants of the mercury(II) complex (K_{31}' and K_{32}') are on the order of 10^2 greater than the corresponding ionization constant (K_{a2}') of cycloserine. If chelation through the amino-group was significant, a much greater increase should have been observed. The fact that the formation constants K_{22}', K_{23}', and β_2' increase successively in value may be accounted for by charge effects as well as by chelation. Similar observations were made by Brooks and Davidson (5) in their analysis of the histidine–mercury(II) system.

Some observations have been made concerning the stability of the cycloserine–metal complexes to hydrolysis. They appear to degrade by a mechanism different from that involved in the decomposition of cycloserine (7). Particularly striking changes may be observed in a solution of the copper (II) chelate upon aging. The solution changes from a green to a deep blue color and deposits a violet precipitate. These observations are the subject of studies now in progress and will be reported on in more detail at a later date.

TABLE II.—COMPARISON OF APPARENT FORMATION CONSTANTS (LOG β_2') OF *d*-CYCLOSERINE AND *dl*-SERINE METAL COMPLEXES

Ligand	Temp., °C.	Hg(II)	Cu(II)	Zn(II)	Co(II)
d-Cycloserine	25	17.5	9.7	6.0	5.7
dl-Serine	20	17.5	14.6	8.6	8.0

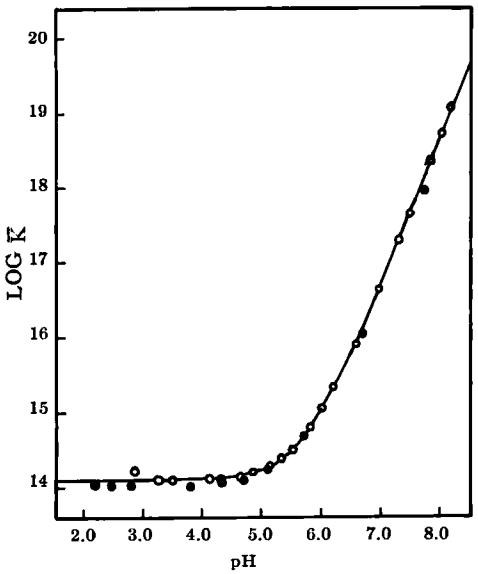


Fig. 1.—Log $\bar{K}$ *vs.* pH: the solid line represents $\bar{K}$ calculated from Eq. 5 using values of $K_{20} = 10^{2.73}$, $K_{21} = 10^{8.67}$, and $K_{22} = 10^{14.07}$; the open circles represent values calculated from measured mercuric ion activities where ΣL is 0.04 *M* and Σ Hg(II) is 0.0004 *M*; the solid circles represent values calculated from measured mercuric ion activities where ΣL is 0 01 *M* and Σ Hg(II) is 0.001 *M*.

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Basis of Hydrogen Ion Binding Curves Deduced from Differences in Solution and Solvent Titrations

By EDWARD R. GARRETT†

A hydrogen ion binding curve may be constructed by plotting the difference in titers of samples and blanks against pH. The theoretical bases for the determination of pKa and stoichiometry for weak acids and bases by such curves is investigated. The assumptions and limitations of such methods are specified. The nature and degree of the difference in the titration curve and the hydrogen ion binding curve is shown for changes in pK and concentration of solute.

IN A PAPER discussing the use of apparent dissociation constants (Ka′) in qualitative organic analysis, Parke and Davis (1) had proposed the use of hydrogen ion binding curves for estimation of pKa′. Their procedures involved the titration of two solutions, one including and one omitting the sample, with titrant of the same high normality. The amount of titer necessary to achieve a given pH for the blank is subtracted from the amount necessary to achieve the same pH for the solution. These titer differences may be calculated in an acidic or basic direction using the amount of titrant at pH 7 as zero for blank and solution. For example, a method would be to subtract the volume of standard acid necessary to bring the blank solution (initially at pH 7) to a given pH, *e.g.*, pH_1, which is less than 7, from the volume of standard acid necessary to change the pH of the sample solution from 7 to that same pH_1. Similarly, the volume of standard alkali necessary to bring the blank solution to a given pH, *e.g.*, pH_2, which is greater than 7, would be subtracted from the volume of standard alkali necessary to change the pH of the sample solution from 7 to that same pH_2.

The titer differences when divided by the total stoichiometric titer for a single titratable function (α) were plotted against the pH and are presumably related to the expression

$$pH = pKa' + \log [\alpha/(1 - \alpha)] \quad \text{(Eq. 1)}$$

where α is the degree of dissociation. The pKa′ was estimated from the pH value at $\alpha = 0.5$ (2).

It should be informative to investigate the basic principles validating this technique and to evaluate its limitations.

The general equation for equilibria during titration of a weak acid (HA) by a base (MOH) on the Parke-Davis assumptions of concentrations equal to activities [or for neutralization of a salt (MA) by a strong acid (HX)] (2) is

$$[H^+] = Ka' \frac{[HA]}{[A^-]} = Ka' \frac{(a - b) - [H^+] + [OH^-]}{b + [H^+] - [OH^-]} \quad \text{(Eq. 2)}$$

where the total concentrations of weak acid and weak acid anion is

$$a = [HA] + [A^-] \quad \text{(Eq. 3)}$$

where the concentration of the anion given as the salt is

$$b = [M^+] \quad \text{(Eq. 4)}$$

and since the solution is electrically neutral

$$[M^+] + [H^+] = [A^-] + [OH^-] \quad \text{(Eq. 5)}$$

Titration of a Salt of a Weak Acid in Acid Region With a Strong Acid.—When pH < 7, $[H^+] \gg [OH^-]$ so that Eq. 2 reduces to

$$[H^+] = Ka' \frac{(a - b) - [H^+]}{b + [H^+]} \quad \text{(Eq. 6)}$$

If x is the number of milliequivalents of MA before titration in L ml. of solution titrated with M ml. of strong acid of y normality, and if M_x is the stoichiometric acid titer for x meq. of MA, then

$$a = x/(L + M) = M_x y/(L + M) \quad \text{(Eq. 7)}$$

and

$$b = (M_x - M)y/(L + M) \quad \text{(Eq. 8)}$$

If M' is the ml. of strong acid titer necessary to achieve the same pH or $[H^+]$ with the same equipment for L' ml. of a blank as M ml. does for L ml. of sample solution, then

$$[H^+] = M' y/(L' + M') \quad \text{(Eq. 9)}$$

Equations 7, 8, and 9 may be substituted into Eq. 6, and since the y values cancel

$$[H^+] = Ka' \frac{\alpha'}{(1 - \alpha')} \quad \text{(Eq. 10)}$$

where

$$\alpha' = \left\{ M - M' \left(\frac{L + M}{L' + M'} \right) \right\} \Big/ M_x \quad \text{(Eq. 11)}$$

The logarithmic form of Eq. 10 is the same as Eq. 1 except for the sign of the log term. The α' values may be calculated for a given pH from knowledge of the ml. of titer (M and M') necessary to achieve that pH in L ml. of sample and L' ml. of the blank.

If the initial volumes of sample and blank solutions are the same ($L = L'$) and the concentration of the acid titer is great with respect to that of the sample solution ($L \gg M > M'$) then the logarithmic form of the simplified expression is

$$pH = pKa' - \log \frac{(M - M')/M_x}{1 - (M - M')/M_x} = pKa' - \log \frac{\alpha''}{1 - \alpha''} \quad \text{(Eq. 12)}$$

Addition of a Strong Acid to a Weak Acid in Acid

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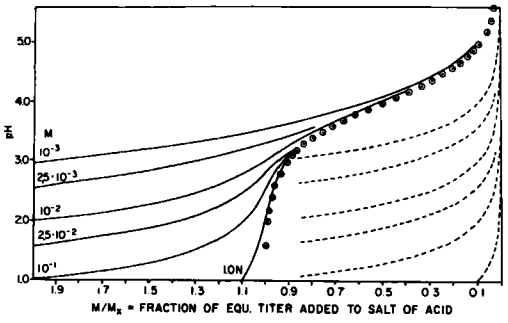

Fig. 1.—Titration and hydrogen ion binding curves for various concentrations of an acid of pKa 4.0; ———, acid titration curves for the weak acid salt on the assumption of no change in concentration of the solution during the titration; – – –, comparable titrations of blank of the same volume; ⊙⊙⊙, difference in titer at any pH for the titration of a sample and blank. It should be noted that the circles give the same S-shaped curve for any concentration of the acid when the titrations are thus corrected for the titration of the blank.

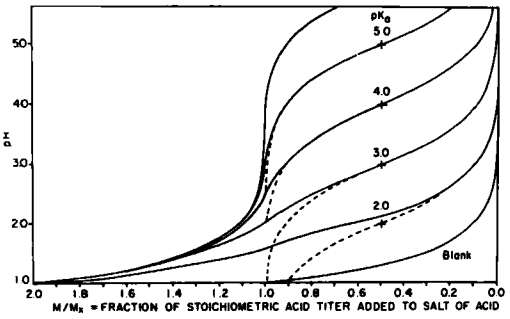

Fig. 2.—Titration and hydrogen ion binding curves for 0.1 *M* solutions of acids of various pKa; ———, acid titration curves for the weak acid salt on the assumption of no change in concentration of the solution during the titration; – – –, difference in titer at any pH for the titrations of sample and blank. The blank is of the same volume as the sample.

Region.—At any time during the addition of a strong acid (HX) to a weak acid (HA), the hydrogen ion concentration $[H^+]$ should be equal to the sum of those ions from the completely dissociated strong acid $[H_s^+]$ and the incompletely dissociated weak acid $[H_w^+]$

$$[H^+] = [H_w^+] + [H_s^+] \qquad \text{(Eq. 13)}$$

However, the acid anion concentration is

$$[A^-] = [H_w^+] = [H^+] - [H_s^+] \qquad \text{(Eq. 14)}$$

and thus,

$$[HA] = a - [H_w^+] = a - [H^+] + [H_s^+] \qquad \text{(Eq. 15)}$$

$$[H^+] = Ka\,\frac{a - [H^+] + [H_s^+]}{[H^+] - [H_s^+]} \qquad \text{(Eq. 16)}$$

Substitution of Eqs. 7, 9 and

$$[H_s^+] = (M - M_x)y/(L + M) \qquad \text{(Eq. 17)}$$

into Eq. 16 results in Eq. 10, 11, and on simplification. to Eq. 12.

Similar equations result for the same assumptions although the sign of the log term in Eqs. 10 and 12 would differ, if the titrations of both blank and sample were carried out in the alkaline range where $[OH^-] \gg [H^+]$, where the undissociated weak acid of the sample is neutralized by strong base and where the pH of the blank is increased by strong base and from Eq. 2

$$[H^+] = Ka'\,\frac{[HA]}{[A^-]} = Ka'\,\frac{(a - b) + [OH^-]}{b - [OH^-]} \qquad \text{(Eq. 18)}$$

Applications of the Derived Equation.—The simplified expression (Eq. 12) states that a difference in titer necessary to achieve the same pH for a sample and a blank solution when divided by the total ml. of stoichiometric titer may be plotted against pH to give an S-shaped curve that is always of the same form.

A series of curves are given in Fig. 1 for the strong acid titrations of various concentrations of a weak acid salt (pKa = 4.0) and their corresponding blanks. The circles represent the ideal S shape calculated for all concentrations on the basis of the assumptions of Eq. 12. The solid lines represent the titration curves of the sample, and the dashed lines the titration curves of the blanks. The estimates of pKa' at $\alpha'' = 0.5$ are practical in a manner equivalent to the use of the pH at half-neutralization as the pKa' (2). This presupposes that the purity and stoichiometry are known for all concentrations of the anion of the acid of pKa 4.0. It must be realized, however, that the assumptions of $M \ll L$, and activity equal to concentration will not hold for very concentrated solutions of the sample.

At low sample concentrations, the error in calculated titer differences will be large.

A series of theoretical curves are also given in Fig. 2 for the strong acid titrations of 0.1 *M* solutions of weak acid salts of various pKa's and a corresponding blank. The dashed lines represent the ideal S shapes calculated on the basis of the assumptions of 12 from the differences in titer of sample and blank solutions where the abscissa for the dashed lines now becomes $(M - M'/M_x)$. The method has no advantage over the traditional techniques of determining pKa' at half neutralization for pKa's generally in the range 4–10, unless solubilities of material are low and the inflections are not sharp. The pKa's of material with $2 < pKa < 4$ may be estimated but the error in subtraction of volumes and in pH readings at pH <2.0, and the failure of the assumptions underlying Eq. 12 invalidate the techniques in those regions, especially for estimates of stoichiometry.

The above discussion considers aqueous solutions. Titrations in nonaqueous solvents are special cases and must be considered separately. For example, a pKa' calculated from the apparent pH at half neutralization in nonaqueous solvent is highly concentration dependent (3).

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Method for Isolation and Determination of α-Methyl DOPA Ethyl Ester in Aqueous Solution

By ARNOLD D. MARCUS and JOSEPH D. DEMARCO

The ethyl ester of α-methyl-3,4-dihydroxyphenylalanine can be quantitatively separated from the free acid by column partition chromatography. Best results have been achieved by using a pH 4.5, 0.1 *M* citrate buffer adsorbed on powdered cellulose as the stationary phase and *n*-butanol as the mobile phase. If the eluate is analyzed colorimetrically using a reagent specific for intact catechol nuclei, interference by any oxidized ester is avoided. The method has been found useful for accelerated stability studies and provides recoveries averaging 99 per cent of theory.

IN AQUEOUS solution the ethyl ester of α-methyl-3,4-dihydroxyphenylalanine (α-methyl DOPA ethyl ester) is subject to two modes of degradation. The ester function may hydrolyze to yield the free amino acid, and the catechol nucleus is, as would be expected, quite prone to oxidation. In view of the two degradative pathways, initial study of the stability of the ester in aqueous solution did not seem amenable to straightforward functional group analysis. Instead, it seemed most desirable to attempt to preferentially isolate the totally intact ester and to conveniently determine its concentration by simple ultraviolet spectrophotometry.

In the event that such complete separation was found too difficult or time consuming, it was felt that isolation of the oxidized and unoxidized ester *together* would be entirely satisfactory if followed by an analytical procedure specific for the intact catechol nucleus.

The main problem, then, concerned separation of the intact or oxidized ester from any hydrolysis products. Toward this end, partition chromatography using suitably buffered and stabilized columns seemed a desirable approach.

Preliminary experiments indicated that successful separation of the ester could be achieved by using columns buffered at a pH of about 5. It was quite evident that the stationary phase should preferably contain both sodium bisulfite and ethylenediamine tetraacetic acid to prevent oxidation of the ester during column residence. Separation of the ester was most easily achieved by elution with *n*-butanol saturated with buffer.

The choice of a holding material was somewhat vexing in that silicic acid columns gave a very slow rate of flow. While columns prepared with Celite 545[1] seemed quite suitable, it was soon apparent that the trace metal contaminants in the Celite promoted oxidation of the ester. Fortunately, columns prepared with powdered cellulose were found entirely acceptable if stored no longer than one week prior to use.

EXPERIMENTAL

Reagents.—pH 4.5, 0.1 *M* citrate buffer; chloroform, Merck reagent; *n*-butanol, Merck reagent; sodium bisulfite; ethylenediamine tetraacetic acid; and powdered cellulose, chromatography grade.

Apparatus.—Chromatographic columns, 40 cm. long × 2 cm. i.d., constricted at the end and provided with a delivery tube to fit into the neck of a 100-ml. volumetric flask.

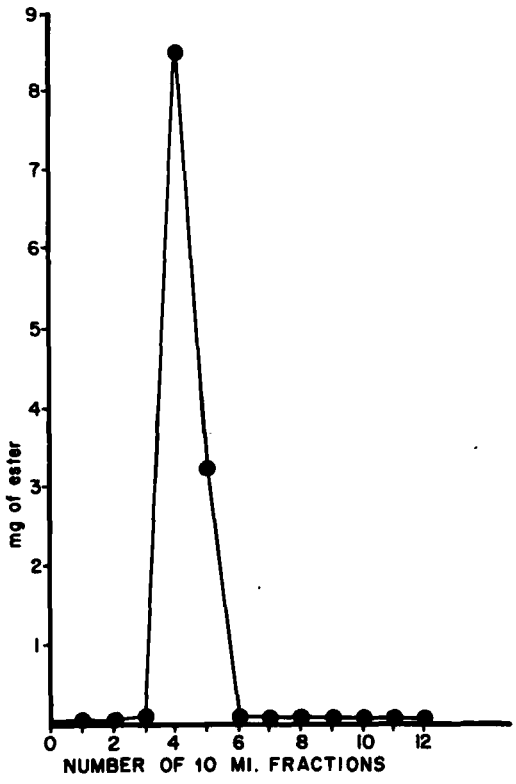

Fig. 1.—Chromatogram showing the elution of α-methyl DOPA ethyl ester from a cellulose column with *n*-butanol.

Procedure.—Sixteen grams of powdered cellulose was mixed mechanically with *ca.* 150 ml. chloroform. Twelve milliliters of buffer previously equilibrated against *n*-butanol and containing 0.35% sodium bisulfite and 0.05% ethylenediamine tetraacetic acid was then added to the cellulose-chloroform mixture with vigorous agitation. The

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[1] Tradename for mineral filler marketed by Johns-Manville Sales Corp., New York, N. Y.

resulting slurry was packed into a column incrementwise using moderate pressure.

Prior to adding the sample, the column was washed with 50 ml. *n*-butanol previously equilibrated against buffer. When the last traces of butanol had entered the column, 1 ml. of a solution of α-methyl DOPA ethyl ester (5–10 mg./ml.) in water or buffer was added to the column and allowed to drain into the body of material. Five milliliters of *n*-butanol was then added to wash the walls of the column and followed by a second 5 ml. of *n*-butanol.

Twenty-five milliliters of *n*-butanol saturated with buffer was then added, and a constant head maintained on the column. At this point, collection of the effluent was started and either small fractions or gross volumes were collected.

Initially all assays were done spectrophotometrically by diluting the effluent with 0.1 *N* methanolic hydrochloride. Subsequently, when it appeared that small amounts of oxidation products were being eluted, all determinations were made by a modification of the procedure of Heinrich and Schuler (1). The procedure used was: the eluate, collected in a volumetric flask, was diluted with 5 *M* acetic acid to provide a concentration of 10 to 15 mcg. of ester per ml. A 5-ml. aliquot of this dilution was transferred to a 25-ml. volumetric flask along with 5 ml. of a 0.6% solution of 4-nitro-2-chloro-1-diazophenyl-β-naphthylsulfonic acid in 5 *M* acetic acid. The mixture was brought to volume with 5 *M* acetic acid and heated at 35° for 1 hour. The flasks were removed from the constant temperature bath, cooled rapidly, and read at 410 mμ *vs.* a suitable reagent blank. In the concentration range employed, all colors showed excellent adherence to Beer's law.

The reagent in 5 *M* acetic acid is stable for at least a week if kept cold. The partition columns should not be kept longer than 1 week.

DISCUSSION OF EXPERIMENTAL RESULTS

When 10 mg. each of α-methyl DOPA ethyl ester and the free amino acid were applied to separate cellulose columns and the effluent collected in 10-ml. fractions, no elution of the free acid could be detected after a total of 25 fractions had been assayed (ultraviolet). In contrast, elution of the ethyl ester was very easily accomplished in a reasonably narrow band as shown in Fig. 1. Although it has been found consistently that all of the ester is eluted in the third through the sixth fraction, we found it simpler to initiate collection of the effluent in a 100-ml. volumetric flask and to collect a total of 100-ml. of effluent. The slight dilution caused by collection of the larger volume is insignificant in view of the high sensitivity of the colorimetric method.

The recoveries of α-methyl DOPA ethyl ester obtained by the method outlined are given in Table I. It is evident from these data that recoveries are well within the limits desired for stability studies and that both the precision and accuracy are satisfactory.

TABLE I.—RECOVERY OF α-METHYL DOPA ETHYL ESTER AFTER PARTITION CHROMATOGRAPHY AND COLORIMETRIC ASSAY

Mg. Added	Mg. Found	% Theory Found[a]
2.56	2.60	102
5.05	4.95	98
9.84	9.84	100
10.00	9.95	99.5
10.67	10.13	95

[a] Average recovery = 98.9% of theory.

Subsequent experiments, in which known quantities (*ca.* 5 mg. each) of α-methyl DOPA ethyl ester and the free acid were chromatographed on single columns, showed the amino acid to have no effect on elution of the ester. Similarly, artificially degraded solutions of α-methyl DOPA ethyl ester (air oxidation at 60°) showed substantial loss after chromatography and colorimetric assay.

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Thin-Layer Chromatography in Drug Analysis II. Procedure for the Identification of Various N^4-Substituted Sulfonamides

By B. T. KHO and SHELDON KLEIN†

A thin-layer chromatographic procedure using the step technique is described for the separation and identification of various N^4-acylated and N^4-aroylated sulfonamides. The test has been successfully applied to mixtures of (*a*) phthalylsulfacetamide, phthalylsulfathiazole, and succinylsulfathiazole and (*b*) phthalylsulfanilamide, succinylsulfacetamide, and succinylsulfanilamide. Detection was accomplished by use of ultraviolet observation and an 0.05% alcoholic bromcresol purple solution. The procedure can be completed within two and one-half hours.

A METHOD for the detection of N^4-acylated and N^4-aroylated sulfonamides alone or when present in mixtures along with N^4-unsubstituted compounds has not yet been reported in the thin-layer chromatography (TLC) literature. Sophoulis (1) has reported a paper chromatographic procedure for various sulfonamides including phthalylsulfacetamide, phthalylsulfathiazole, and succinylsulfathiazole, which required 6 hours for development. The procedure gave R_f values which were too close to provide separation of phthalylsulfacetamide and succinylsulfathiazole

The usual color reactions for the detection of the unsubstituted sulfonamide spots are based on the reaction of the free aromatic NH_2 group. The standard procedure for the detection of N^4-substituted sulfonamides on paper is by use of acid-base indicators (2). In developing the TLC procedure described in this paper, it was found that for certain substituted compounds, bromcresol purple gave the best results when silica gel G was used. For others, detection by ultraviolet fluorescence was necessary.

This paper deals with the separation and identification, by TLC, of the N^4-acylated and N^4-aroylated sulfonamides together or in combination with N^4-unsubstituted sulfonamides. A successful procedure was obtained using the step technique (3). This necessitated the use of two chambers. One of the two was lined with filter paper that was saturated with the solvent system. This was necessary to avoid the edge phenomenon (3). Unsubstituted sulfonamides are detected on the same plates using the Bratton-Marshall reagent. The identification of these was accomplished according to a previously described method (4).

The procedure is useful in the identification of the N^4-substituted sulfonamides present in the U.S.P. XVI (5) and N.F. XI (6). It also provides a means for the detection of the corresponding unsubstituted sulfonamides, if present.

EXPERIMENTAL

Apparatus.—Glass plates (200 × 200 mm.). An unlined tank ($8^1/_2$ in. × 4 in. × $8^1/_2$ in.) for solvent A. A second tank similar to A but containing solvent B and lined with solvent-saturated filter paper.

Preparation of Plates.—The plates were coated according to the procedure described in the previous paper (4).

Solvent A.—Methanol-ethanol (1:1). Prepared by mixing 60 ml. each of anhydrous ethanol and A.C.S. reagent methanol.

Solvent B.—*n*-Propanol–0.05 *N* hydrochloric acid (4:1). Prepared by mixing 100 ml. of *n*-propanol, 25 ml. of water, and 0.1 ml. of concentrated hydrochloric acid.

Adsorbant.—Silica gel G.

Standards.—Twenty-five mg. dissolved in 50 ml. acetone. These were freshly prepared. The compounds used were: phthalylsulfacetamide (I),[1] phthalylsulfathiazole (II), succinylsulfathiazole (III), phthalylsulfanilamide (IV),[1] succinylsulfanilamide (V),[1] and succinylsulfacetamide (VI)[1].

Preparation of Samples.—An equivalent of 25 mg. of sulfonamide was extracted with 50 ml. of acetone. For suspensions, 1 ml. of water was added before extraction. If necessary, the solutions were centrifuged and the clear supernatant liquid used.

Observation.—Short wave U.V. lamp (254 mμ)[2] and 0.05% alcoholic solution of bromcresol purple.

Quantity of Sulfonamide Applied.—One mcg. for compounds I to IV inclusive, 3 mcg. for compounds V and VI.

Using the Desaga apparatus,[3] the adsorbant was applied to the plates as described in the previously published paper (4). The tanks were allowed to equilibrate overnight. In addition to a finish line at 100 mm. from the start, a small notation was made at the 50-mm. level.

Samples (2 μl.) and standard (2 μl.) solutions representing compounds I to IV inclusive were applied on the plates along an imaginary line 15 mm. from the starting edge. For compounds V and VI, 6 μl. was applied. Two microliters of a standard solution containing 1 mg. per 2 ml. and representing a possible unsubstituted hydrolysis product were also applied.

Development was started in tank A. When the front reached 50 mm., the plates were removed, dried at 100° for 5 minutes, allowed to cool, and then placed into tank B. When final development to 100 mm. was completed, the plates were removed and again dried at 100° for 5 minutes. Observation was begun using a short wave U.V. lamp.

Phthalylsulfacetamide (I), Phthalylsulfathiazole

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[1] Obtained from K and K Laboratories, Jamaica 33, N. Y.

[2] Chromato-Vue C-3 marketed by Black Light Eastern Corporation, 4 Manhasset Ave., Port Washington, L. I. N. Y., or the equivalent.

[3] Obtainable from C. Desaga, Heidlberg, Germany, through Brinkmann Instruments, Inc., Great Neck, L. I., N. Y.

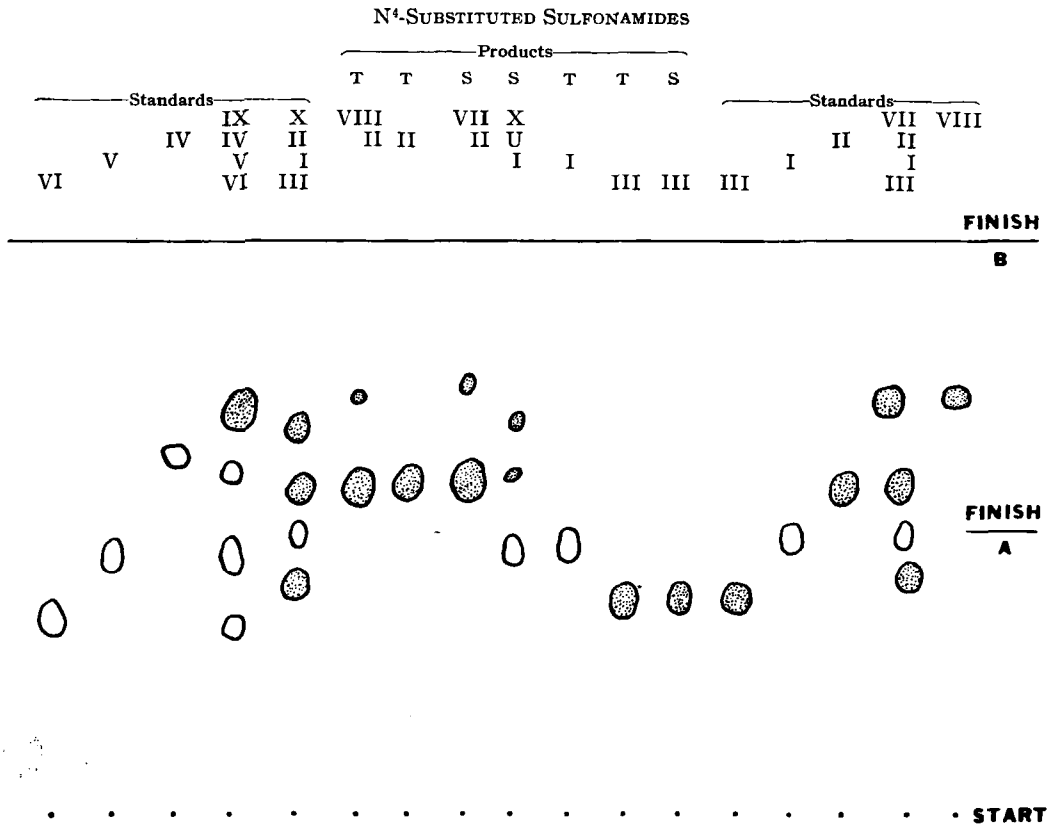

Fig. 1.—I = Phthalylsulfacetamide; II = phthalylsulfathiazole; III = succinylsulfathiazole; IV = phthalylsulfanilamide; V = succinylsulfanilamide; VI = succinylsulfacetamide; VII = sulfathiazole; VIII = sulfamethazine; IX = sulfanilamide; and X = sulfacetamide. U, unknown; A, solvent A; B, solvent B; T, tablet; and S, suspension. The clear spots refer to areas detected by use of ultraviolet lamp. The shaded spots refer to areas detected by use of bromcresol purple indicator.

(II), and Succinylsulfathiazole (III).—Compound I appeared as a bright blue fluorescent area which was marked, if present. Compounds II and III sometimes appeared faintly blue. Spraying with 0.05% alcoholic solution of bromcresol purple showed blue or purple spots on a yellow background in the presence of II and III or any unsubstituted sulfonamide produced by hydrolysis. Compound I appeared blue when first sprayed, but the color faded with either continued spraying or within a short period after the spraying was completed. On first application, the entire plate appeared blue. The background rapidly faded, leaving only areas of II, III, and the unsubstituted sulfonamides visible.

A reproduction of a developed plate is shown in Fig. 1. The copy was obtained using a Xerox 914 copier and a modified procedure of Hilton and Hall (7).

Phthalylsulfanilamide (IV), Succinylsulfanilamide (V), and Succinylsulfacetamide (VI).—The sensitivity of the U.V. detection method for observing V and VI is less than for any of the other four compounds tested. A 3 mcg. sample was the smallest amount easily detected. Compounds V and VI appeared as blue fluorescent areas after exposure of the plates to the ultraviolet lamp for a period of two or three minutes. Spraying with the indicator gave rapidly fading blue or purple spots.

A summary of approximate R_f values for the various sulfonamides tested is shown in Table I.

TABLE I.—APPROXIMATE R_f VALUES OBTAINED FOR VARIOUS N⁴-SUBSTITUTED SULFONAMIDES

Sulfonamide	Av. R_f[a]	Observation
Succinylsulfacetamide	0.37	UV
Succinylsulfathiazole	0.43	Indicator
Succinylsulfanilamide	0.49	UV
Phthalylsulfacetamide	0.51	UV
Phthalylsulfathiazole	0.61	Indicator
Phthalylsulfanilamide	0.66	UV

[a] Data obtained from seven or more plates.

RESULTS AND DISCUSSION

For the samples tested, separation and identification of the N⁴-substituted sulfonamides was accomplished by use of the step technique (3). The substituted compounds were easily separated from the free sulfonamides. Verification of the presence of any unsubstituted sulfonamides can be made using diazotization and the Bratton-Marshall reagent. For identifying the unsubstituted sulfon-

amide, the method described in the previously published paper (4) was used.

The R_f values for the N^4-substituted sulfonamide official in the U.S.P. XVI (5) and N.F. XI (6), namely I, II, and III were sufficiently different to permit identification

The R_f values for IV, V, and VI were also sufficiently different to allow separation from each other. Of the six compounds tested, all can be separated either by R_f values or fluorescent observation, except I and V. At equal concentrations of 1 mcg. per spot, V is not visible. Though II and IV give close R_f values, II changes the indicator color to blue or purple whereas IV does not. Compound IV gives a bright blue fluorescence and II does not.

After two days the standard solutions showed the presence of the free form.

Due to the uncertainty of reproducing R_f values from one plate to another, it is important to use standards along with the samples.

The total development time is approximately two and one-half hours.

REFERENCES

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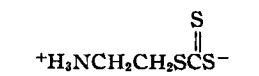
Communications

Structure of the Carbon Disulfide Adduct of β-Mercaptoethylamine

Sir:

We have recently reported (1, 2) the preparation of a compound from the reaction of β-mercaptoethylamine (MEA) and carbon disulfide, in either aqueous or alcoholic ammonia, which gave good protection to animals against an otherwise lethal dose of X-irradiation. This compound was believed to be 2-mercaptoethyldithiocarbamic acid on the basis of elemental analysis, its reaction with acylating agents, and a characteristic dithiocarbamate absorption peak (3) in the ultraviolet at 248 mμ (log ε max. = 3.03) (C_2H_5OH). Other characteristic absorption peaks for dithiocarbamates at 290–310 mμ and 340–360 mμ were absent, however, and further examination of the ultraviolet absorption characteristics of dithiocarbamate and trithiocarbonate zwitterions has shown the product in question to be a trithiocarbonate zwitterion I. A previous example of a trithiocarbonate zwitterion has not appeared in the literature.



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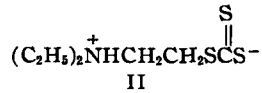

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Trithiocarbonate zwitterion II of unquestioned structure was prepared from 2-diethylaminoethanethiol hydrochloride (Evans Chemetics, Inc.) and carbon disulfide in ammoniacal solution, analogously to the preparation of I, m.p. 114–115° with effervescence, (90% yield).

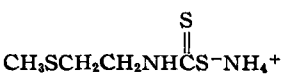
Anal.—Calcd. for $C_7H_{15}NS_3$: C, 40.16; H, 7.22; N, 6.70; S, 45.94. Found: C, 40.74; H, 7.20 N, 6.96; S, 46.13.

The compound reduced iodine, and it showed definite trithiocarbonate absorption peaks (4) in the ultraviolet at 226 mμ (log ε max. = 4.07) and 303 mμ (log ε max. = 4.18) in alkaline C_2H_5OH. Compound I, quite similar in color and physical appearance, showed ultraviolet absorption peaks at 226 mμ (log ε max. = 3.96) and 304 mμ (log ε max. = 4.15), when observed in the same solvent.

Furthermore, compound III, possessing definite dithiocarbamate absorption characteristics in the ultraviolet, was prepared from the S-methyl ether of MEA [prepared by the procedure of Gonick (5)] and carbon disulfide in ammoniacal solution, and was isolated as an unstable ammonium salt, m.p. 104–106°, (70% yield).

Anal.—Calcd. for $C_4H_{12}N_2S_3$; N, 15.27; S, 52.17. Found: N, 15.06; S, 51.38.

Loss of ammonia and carbon disulfide was evident within 24 hours. Characteristic dithiocarbamate absorption was observed at 257 mμ (log ε max. = 3.92), 292 mμ (log ε max. = 4.06), and 345 mμ (log ε max. = 1.95) in alkaline C_2H_5OH.



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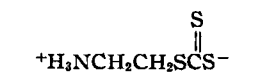

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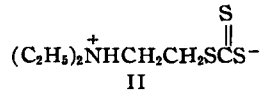

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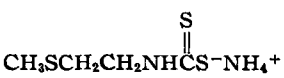

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Alkaloids of *Vinca rosea* Linn. (*Catharanthus roseus* G. Don) XVIII. Root Alkaloids

Sir:

A number of crude amorphous alkaloidal fractions from root extracts had demonstrable anti-P-1534 leukemia activity in DBA/2 mice, and it was deemed necessary to determine whether this oncolytic activity was caused by any of the four known dimeric alkaloids or by any new entities. While the roots had been examined previously by other investigators (1), they had not been looked at utilizing the method of selective extraction, followed by column chromatography and gradient pH techniques as devised in this laboratory (2, 3).

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The *B* fraction gave three new alkaloids, ammocalline,[1] pericalline, and ammorosine, as well as akuammicine [first reported as being obtained from *Picralima klaineana* Pierre (6)], along with lochnerivine, leurosine, perivine, virosine, and lochnerine.

Pertinent physical data of these new alkaloids are as follows:

Vinosidine crystallized from methanol, m.p. 253–257° dec.; pK'a = 6.80 (33% DMF).

Anal.—Calcd. for $C_{22}H_{26}N_2O_5$: C, 66.31; H, 6.58; N, 7.03; O, 20.08. Found: C, 66.26; H, 6.72; N, 6.75; O, 20.51.

The ultraviolet spectrum in ethanol is characterized by maxima at 226, 254, 259, and 300 mμ, with a shoulder at 330 mμ; log E (1%, 1 cm.) = 3.01, 2.46, 2.46, 2.20, and 1.90, respectively. The infrared spectrum of a $CHCl_3$ solution exhibits the following 11 characteristic bands, listed in the order of decreasing intensities (as in all following examples): 1220, 1299, 1266, 1282, 1538, 1695, 1149, 1136, 1724, 1471, and 1087 $cm.^{-1}$

Lochnerivine crystallized from methanol, m.p. 278–280°; no titratable groups were found between pH 3.0–11.0.

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The base, isolated from the sulfate, would not yield to crystallization from any of the solvents used. Its ultraviolet spectrum in ethanol is identical to that of the sulfate. The infrared spectrum of a $CHCl_3$ solution shows the following bands: 1231, 1738, 1456, 1041, 1427, 1495, 1168, 1612, 1366, 1143, and 1001 cm.$^{-1}$.

Cavincine crystallized as the sulfate from ethanol, m.p. 275–277° dec.; pK'a = 6.90 (33% DMF). The ultraviolet spectrum in ethanol is characterized by maxima at 224, 281, and 288 mμ; log E (1%, 1 cm.) = 2.86, 2.17, and 2.08, respectively. The infrared spectrum of a Nujol mull shows the following bands: 1453, 1725, 1088, 1022, 1029, 1371, 1051, 1132, 746, 1143, 1131, and 1160 cm.$^{-1}$.

The base, isolated from the sulfate, could not be crystallized. Its ultraviolet spectrum in ethanol is identical to that of the sulfate. The infrared spectrum of a Nujol mull shows the following bands: 1449, 1708, 1372, 737, 1162, 1198, 1222, 1258, 1321, 1348, and 1058 cm.$^{-1}$.

Ammocalline crystallized from acetone, m.p. > 335° dec.; pK'a = 7.30 (33% DMF).

Anal.—Calcd. for $C_{19}H_{22}N_2$: C, 81.97; H, 7.97; N, 10.06. Found: C, 81.63; H, 7.69; N, 9.93.

The ultraviolet spectrum in ethanol is characterized by maxima at 218 and 288 mμ; log E (1%, 1 cm.) = 3.09 and 2.61, respectively. The infrared spectrum of a Nujol mull shows the following bands: 1467, 748, 1435, 737, 1330, 906, 1381, 1340, 1298, 1144, and 1320 cm.$^{-1}$.

Pericalline crystallized from acetone, m.p. 196–202°; pK'a = 8.05 (33% DMF). The ultraviolet spectrum in ethanol is characterized by maxima at 207 and 304 mμ, with shoulders at 230 and 240 mμ; log E (1%, 1 cm.) = 2.98, 2.80, 2.84, and 2.75, respectively. The infrared spectrum of a $CHCl_3$ solution shows the following bands: 1322, 1458, 1120, 1441, 1420, 1230, 1601, 885, 820, 661, and 1300 cm.$^{-1}$.

Ammorosine crystallized from methanol, m.p. 221–225°; pK'a = 7.30 (33% DMF).

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Mitraphylline and akuammicine were both obtained by crystallization from acetone. They were identified by comparison of melting points, X-ray powder diffraction patterns, and infrared and ultraviolet spectra with those of authentic samples.

Detailed methods of obtaining these alkaloids, along with their preliminary characterization, will be discussed at a later date.

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Paper XVII in this series: Gorman, M., and Neuss, N., *J. Am. Chem. Soc.*, in press.

Structure of "2-Mercaptoethyldithiocarbamic Acid"

Sir:

Recently Foye, Duvall, and Mickles (1, 2) have assigned the 2-mercaptoethyldithiocarbamic acid structure to the significantly radioprotective compound derived from reaction of equimolar amounts of 2-mercaptoethylamine (MEA) hydrochloride and carbon disulfide in ammoniacal solution. The chemical and physical properties reported for this material suggested reaction had occurred at the thiol end of the MEA molecule, rather than the amino end, producing the zwitterion of 2-aminoethyltrithiocarbonic acid.

$$HSCH_2CH_2NH_3^+Cl^- + CS_2 + NH_3 \rightarrow {}^-SC(=S)SCH_2CH_2NH_3^+ + NH_4^+Cl^-$$

Repetition of Foye's procedure (1) gave a yellow solid in about 75% yield. Like his material, it was insoluble in most common solvents and unstable in air. When isolated without washing (Foye's procedure) it melted at 80–82° dec. in a capillary. Washing with substantial quantities of water or ethanol raised the melting point without appreciably diminishing the yield or changing the infrared spectrum. In a capillary our test preparation melted at 86–88° dec., but on a block the melting point was much lower, 80–82°, and decomposition was not apparent. Foye reported a melting point of 76–78°, taken on a block. From the method of preparation, solubility behavior, and melting point data we conclude that our compound was identical with that reported by Foye. Our best washed sample (analysis below) was probably somewhat purer than his material.

Anal.—Calcd. for $C_3H_7NS_3$: C, 23.51; H, 4.61; N, 9.14; S, 62.75. Found: C, 23.15; H, 4.67; N, 9.48; S, 62.77.

To obtain physical and chemical evidence bearing on the structure problem it was necessary to find solvents for the compound. The zwitterion hypothesis suggested that highly polar solvents would be most suitable, and indeed the compound was found to be very soluble in dimethyl sulfoxide and dimethylformamide.

The proton nuclear magnetic resonance spectrum, taken in deuterated dimethyl sulfoxide with tetramethylsilane as standard, showed a broad absorption band at 7.00 p.p.m., assignable to $-NH_3^+$, and a multiplet at 3.28 p.p.m., assignable to $-CH_2-$, with relative areas almost exactly 3:4. If present, —SH should have appeared as a well defined triplet at about 1.75 p.p.m., and no such absorption was noted. The n.m.r. spectrum is thus completely consistent with the 2-aminoethyltrithiocarbonic acid zwitterion structure and totally inconsistent with the 2-mercaptoethyldithiocarbamic acid structure.

A solution of the compound in dimethylformamide reacted exothermically with benzyl chloride, and the product, obtained in low yield because of isolation difficulties, was shown by analysis to be the hydrochloride of 2-aminoethyl benzyl trithiocarbonate. This reaction and product are obviously possible only if the starting material is the zwitterion of 2-aminoethyltrithiocarbonic acid.

$${}^-SC(=S)SCH_2CH_2NH_3^+ + C_6H_5CH_2Cl \rightarrow C_6H_5CH_2SC(=S)SCH_2CH_2NH_3^+ Cl^-$$

Anal.—Calcd. for $C_{10}H_{14}NS_3Cl$: C, 42.92; H, 5.04; N, 5.01; S, 34.37; Cl, 12.67. Found: C, 42.66; H, 4.95; N, 5.12; S, 34.12; Cl, 12.61.

Finally, it was reasoned that if reaction had occurred at the thiol end of MEA, it should also be possible with mercaptoamines completely substituted on the nitrogen atom; if it had taken place at the amino end, no reaction should be possible with completely substituted mercaptoamines. To test this concept, equimolar amounts of 2-dimethylaminoethanethiol hydrochloride and carbon disulfide were reacted in the presence of excess aqueous ammonia under Foye's conditions. A yellow solid, m.p. 128–129.5° dec., was obtained in about 70% yield. The physical properties of this material were very similar to those of the MEA-CS_2 product, and its elemental analysis indicated that it resulted from combination of 1 mole of mercaptoamine with 1 mole of carbon disulfide. Since this compound can be formulated *only* as the zwitterion of 2-dimethylaminoethyltrithiocarbonic acid, its ready formation under Foye's conditions lends support to the belief that the MEA-CS_2 product is similarly constituted.

$$HSCH_2CH_2NH^+(CH_3)_2Cl^- + CS_2 + NH_3 \rightarrow {}^-SC(=S)SCH_2CH_2NH^+(CH_3)_2 + NH_4^+Cl^-$$

Anal.—Calcd. for $C_5H_{11}NS_3$: C, 33.12; H, 6.12; N, 7.73; S, 53.04. Found: C, 33.37; H, 6.14; N, 7.49; S, 52.89.

From all this evidence we conclude that the

compound reported by Foye, *et al.* (1, 2), as 2-mercaptoethyldithiocarbamic acid is actually the zwitterion of 2-aminoethyltrithiocarbonic acid. This structure is consistent also with the radioprotective activity shown by the material, since the very similar zwitterions of 2-aminoethylthiosulfuric acid and related compounds also are known to possess significant activity as radioprotectants (3, 4).

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compound reported by Foye, *et al.* (1, 2), as 2-mercaptoethyldithiocarbamic acid is actually the zwitterion of 2-aminoethyltrithiocarbonic acid. This structure is consistent also with the radioprotective activity shown by the material, since the very similar zwitterions of 2-aminoethylthiosulfuric acid and related compounds also are known to possess significant activity as radioprotectants (3, 4).

(1) Foye, W. O., Duvall, R. N., and Mickles, J., THIS JOURNAL, 51, 168(1961).
(2) Foye, W. O., and Mickles, J., *J. Med. Pharm. Chem.*, 5, 846(1962).
(3) Holmberg, B., and Sorbo, B., *Nature*, 183, 832(1959).
(4) Kaluszyner, A., Czerniak, P., and Bergmann, E. G., *Radiation Res.*, 14, 23(1961).

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Book Notices

Inorganic Chemistry. By LOUIS K. SHARP. The Williams and Wilkins Co. (exclusive U. S. agent), 428 East Preston St., Baltimore 2, Md., 1962. viii + 339 pp. 15 × 23 cm. Price $8.

The author has designed this book to meet the requirements of students of pharmacy, medicine, and allied sciences by limiting the discussions of elementary chemistry and concentrating on those subjects of particular importance to medicinal and pharmaceutical chemistry students. The author has increased the treatment of inorganic compounds handled by workers engaged in research and analytical procedures, though not actually used as medicaments, which have particular methods of preparation and purification or peculiar hazards or disposal problems associated with them. The volume is divided into two parts: Part I is concerned with fundamental material and Part II contains factual material on the elements and compounds used as medicaments or as tools in analysis and research.

Elements of General and Biological Chemistry. An Introduction to the Molecular Basis of Life. By JOHN R. HOLUM. John Wiley & Sons, Inc., 440 Park Avenue South, New York 16, N. Y., 1962. ix + 470 pp. 14.5 × 23 cm. Price $5.95.

The molecular basis of life is the general theme of this textbook designed for students who, though not intending to become chemists, will profit from an awareness of the molecular basis of life in their chosen occupations. This book is not a general introductory view of general chemistry; it singles out certain processes of life at the molecular level and treats the selected topics in some depth.

Scientific Research in British Universities and Colleges 1961–62. Department of Scientific and Industrial Research, State House, High Holborn, London, W.C. 1, 1962. U. S. agents: British Information Services, 45 Rockefeller Plaza, New York 20, N. Y. xvii + 622 pp. 13 × 21.5 cm. Price $6.50.

This paperbound volume provides an extensive listing of scientific research in progress in British universities, university colleges, and associated institutions as well as research being conducted in the colleges of advanced technology in England, Wales, Scotland, and Northern Ireland during the 1961–1962 academic year. One hundred and sixteen institutions are included. Under the listing for each institution are brief, title-like descriptions of the projects under study in the various departments along with a list of workers supervising the research. The book is well indexed—a name index, a topic index, and a subject index. In all, the indexes cover about 165 pages of the volume.

Immunoassay of Hormones. Edited by G. E. W. WOLSTENHOLME and MARGARET P. CAMERON. Little, Brown and Co., 34 Beacon St., Boston 6, Mass., 1962. xii + 419 pp. 14 × 20 cm. Price $10.75.

This book is volume 14 of the Ciba Foundation Colloquia on Endocrinology covering a symposium in which 28 scientists, representing both immunologic and endocrinologic thought, participated. The symposia dealt with the complex problem of detection and assay of hormones by immunochemical means. Major topics included in the contents of the volume are: Growth hormone, Insulin, Glucagon, Thyrotropin, Corticotropin, Gonadotropins, and Prolactin. The papers presented and the general discussion which followed the papers should represent an up-to-date review of the status of work in this area. As with previous volumes, author and subject indexes are included.

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The Editor comments

EXCELLENCE IN TEACHING

Each year at about this time we read of various awards being made to university professors or instructors for *excellence in teaching*. This type of award is a relative newcomer among the longer established and more widely known categories of honors. Such awards are intended to single out dedicated teachers, who—through their special classroom technique and personal inspiration—make a significant, favorable impression on their students. And, in so doing, they are able to contribute in a positive and enduring manner to the education and motivation of those students. Selection of the award recipients, at least in part, is often made by those in the best position to judge the teacher's ability—namely, the students themselves.

We have been pleased to note that the number of these annual awards appears to be increasing, and particularly that the number of pharmacy school faculty recipients is growing at perhaps an even faster rate.

Our pleasure in large part stems from the fact that these awards serve to recognize and encourage the many devoted and deserving faculty people who for years were virtually ignored while the spotlight of attention almost exclusively was beamed solely on the research professors and the more dramatic discoveries issuing from their laboratories. It is certainly not our intention to suggest that the tribute paid in recognition of research activities has been either inappropriate or excessive. However, similar attention to the beneficial value of the contributions being made by teachers of outstanding ability has been long overdue. It is gratifying to many of us that this unfortunate neglect is being corrected.

Edward G. Feldmann

JOURNAL OF Pharmaceutical Sciences

May 1963 volume 52, number 5

Review Article

Metabolism of Phenothiazine Drugs

By JOHN L. EMMERSON† and TOM S. MIYA

OVER A PERIOD of years beginning in 1944, the research workers of the Rhône-Poulenc Research Laboratories in France synthesized a number of amine derivatives of phenothiazine. One of the most important of these was chlorpromazine prepared by Charpentier in 1950. This compound was found to exert a remarkable number of pharmacological effects involving the central and autonomic nervous systems. Courvoisier, *et al.* (1), have published an extensive report on the pharmacological activity of chlorpromazine in animals. The most striking aspect of this drug, however, was its therapeutic value in patients with certain emotional disturbances. This discovery provided the initial stimulus for the development and use of phenothiazine drugs in psychiatric practice.

The search for new drugs to treat specific mental disorders and the attempts to isolate a particular pharmacological effect by chemical alteration of the phenothiazine molecule has resulted in the introduction of several useful phenothiazine drugs of varying actions and potencies. These developments offer a unique opportunity, not only for the study of structure-activity relationships, but also for the examination of the effect of different chemical substituents on the biological fate of these compounds, their metabolism, distribution, and excretion.

Following oral or parenteral administration, most of the phenothiazine derivatives are extensively localized in body tissues. This property of the parent compound and/or its metabolites is probably responsible for the unusually prolonged excretion which has been observed (2, 3). The possibility that metabolites may be present in the body for long periods of time underscores the importance of the identification of these compounds and the role they play in the effectiveness of the phenothiazine drugs.

Results of studies on model compounds strongly suggest that the metabolites contribute to the action of the parent drug (4). Clinical changes in psychotic patients, the type of psychiatric disorder, or the rate of regression after drug withdrawal may be correlated with the nature and quantity of the metabolic products found in the urine. These factors have tended to focus attention on the biological fate of the phenothiazine drugs.

This review of the literature and summary of the present state of knowledge concerning the metabolism of the phenothiazine drugs deals primarily with the biological transformation of phenothiazine derivatives currently used as psychotherapeutic agents. The equally important topics of absorption, tissue distribution, and excretion will be considered in a subsequent report. A substantial part of the present review is devoted to the discussion of chlorpromazine metabolism. Chlorpromazine has been used as a representative compound for the following reasons: 1. the preponderance of the relevant literature concerns chlorpromazine; 2. chlorpromazine data have, in many cases, been independently verified; 3. chlorpromazine was the first compound of this series to be examined for metab-

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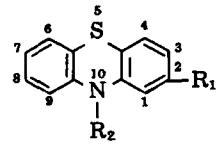
TABLE I.—CHEMICAL STRUCTURES OF SOME PHENOTHIAZINE DRUGS



Compound	R_1	R_2
Chlorpromazine	—Cl	$-CH_2CH_2CH_2N(CH_3)_2$
Methoxypromazine	—OCH_3	$-CH_2CH_2CH_2N(CH_3)_2$
Promazine	...	$-CH_2CH_2CH_2N(CH_3)_2$
Promethazine	...	$-CH_2CH(CH_3)N(CH_3)_2$
Fluphenazine	—CF_3	$-CH_2CH_2CH_2N$ piperazine $N-CH_2CH_2OH$
Perphenazine	—Cl	$-CH_2CH_2CH_2N$ piperazine $N-CH_2CH_2OH$
Thioridazine	—SCH_3	$-CH_2CH_2-$ piperidine $N-CH_3$
Mepazine	...	$-CH_2-$ piperidine $N-CH_3$
Trifluoperazine	—CF_3	$-CH_2CH_2CH_2N$ piperazine $N-CH_3$

olism. A recent review by Bousquet (5) may be consulted for current information in the general area of drug metabolism. The reader is also referred to a review of the metabolism of psychoactive drugs by Carr (6) and an excellent review of the pharmacology of the tranquilizer drugs by Domino (7). The latter paper also discusses the metabolic fate of these compounds. Harwood (8) has reviewed the metabolism of phenothiazine.

In order to facilitate the presentation of data, the literature is discussed under subject headings which relate to a particular type of metabolic reaction. For the sake of brevity and continuity, the generic names of the phenothiazine drugs have been used. Table I lists the generic names and chemical structures. The structural formulas of several known and hypothesized metabolites are given in Table II. Although some later papers are included, this review is chiefly concerned with the literature through August 1962.

SULFOXIDATION

Prior to 1950, the oxidation of organic sulfur compounds to sulfoxides was a rather novel type of biological transformation. Other than the work of Clare, *et al.* (9), on the metabolism of phenothiazine, little was known of this metabolic reaction. Phenothiazine-5-oxide was found in the blood and aqueous humor of the eye in calves which had been treated with the anthelmintic, phenothiazine. Today the accumulated evidence indicates that the formation of the sulfoxide (5-oxide) is a metabolic reaction common to all the phenothiazine derivatives in current use.

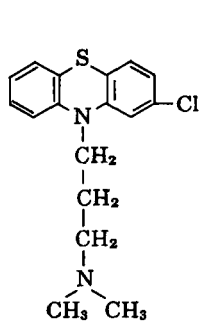
Salzman, *et al.* (10), reported in 1955 that they were able to isolate, by counter-current methods, a major metabolite of chlorpromazine and to identify this compound as the sulfoxide. In studies of urinary excretion (11), this form was found to comprise from 10–14% of the administered dose of chlorpromazine in dogs and about 5% of the dose in man. Only very small amounts of the drug were excreted unchanged by either species. In a study of the urinary excretion patterns of patients receiving daily chlorpromazine medication, Huang and Kurland (12) have shown that the average urinary excretion (*per diem*) of chlorpromazine and its sulfoxides varied from 1–20% of the daily dose. Sulfoxide excretion ranged from 1–18%, while the amount of free chlorpromazine in the urine was usually less than 1% of the dose. The average ratio of free chlorpromazine to its sulfoxides was found to be approximately 1:16.

Fishman and Goldenberg (13), by chromatographic studies, were able to show ten metabolites of chlorpromazine which were extractable at an alkaline pH from human urine. Six of these compounds gave ultraviolet spectra characteristic of sulfoxides. In addition to chlorpromazine sulfoxide, two demethylated sulfoxides were shown by chromatography. In subsequent

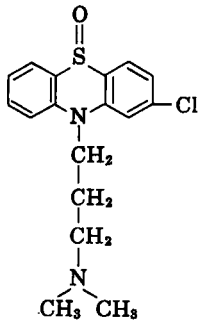
studies, these workers were able to quantify some of the urinary metabolites of chlorpromazine in man and dog (14). Values for total sulfoxide excretion were given as 5.9% of the dose in man and 11.3% in the dog. These figures agree closely with the earlier findings of Salzman and Brodie (11). It should be pointed out that excretion data for sulfoxides, as reported by Goldenberg and Fishman (14), were calculated as the sum of three different sulfoxides separated by chromatography and determined individually. Since the colorimetric assays in earlier reports gave no indication of an alteration in the side chain, the quantities given for a sulfoxide metabolite probably represented some demethylated products.

Sulfoxides have been found to be the principal metabolic products of chlorpromazine in the mouse. Upon administration of S^{35}-labeled chlorpromazine to mice, the radioactivity in the

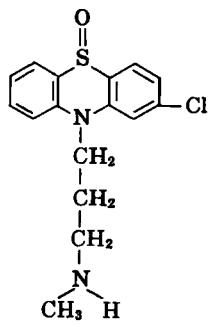
TABLE II.—SOME EXAMPLES OF REPORTED METABOLIC REACTIONS OF PHENOTHIAZINE DRUGS



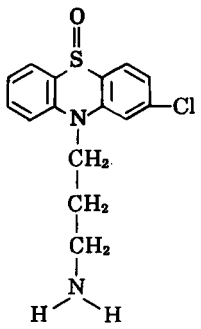
Chlorpromazine



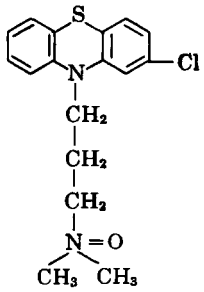
Chlorpromazine sulfoxide



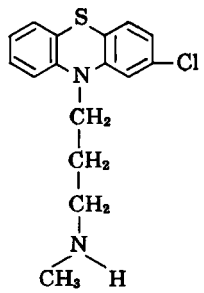
Desmethyl chlorpromazine sulfoxide



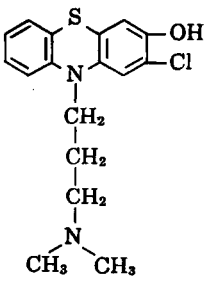
Desdimethyl chlorpromazine sulfoxide



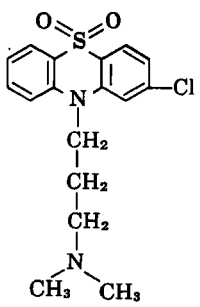
Chlorpromazine-N-oxide



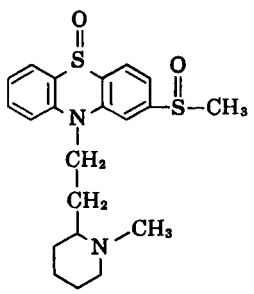
Desmethyl chlorpromazine



3-Hydroxy-chlorpromazine[a]



Chlorpromazine sulfone[a]



Thioridazine disulfoxide

[a] Hypothetical metabolites of chlorpromazine.

urine was found in the forms of free sulfate (3–5%), "combined sulfate" (23–48%), and the sulfoxide of chlorpromazine (49–73%) (15). In the rat, Ogawa, *et al.* (16), found that 27% of the dose of chlorpromazine appeared in the urine within 72 hours. The unchanged drug accounted for 8.1% of the dose while 18.9% was present as the sulfoxide. Other studies have shown that approximately 40% of the administered radioactivity is excreted by the rat in the urine when S^{35}-chlorpromazine is given (17, 18). Emmerson and Miya (18) were able to account for 12% of the dose in the urine as unmetabolized drug and a similar amount as sulfoxides of chlorpromazine.

Tissue homogenates have been used to study the oxidation of chlorpromazine *in vitro*. Chlorpromazine sulfoxide has been isolated from the incubation media. The ability to metabolize chlorpromazine is apparently centered in the liver, for other tissues have shown little activity in this respect (19, 20).

By intravenous administration of chlorpromazine sulfoxide to dogs, it has been shown that the sulfoxide itself is metabolized. Less than one-quarter of the original dose was excreted unchanged, and chromatography of the urine revealed a further metabolic product of the sulfoxide (11). Similar results have been reported in man (21). The biological half-life of the sulfoxide in human plasma has been calculated to be about 3 hours. The duration of its sedative effect was short; it usually lasted not more than 1 hour. Following an intravenous dose to patients, only 2–3% of the dose could be recovered as unchanged drug during the subsequent 24 hours (21).

In vitro studies have provided further evidence that chlorpromazine sulfoxide undergoes additional metabolism (19, 20). It has been postulated that chlorpromazine sulfone (chlorpromazine-5-dioxide) may be one of the *in vitro* products (20). Chlorpromazine sulfone has not been encountered in the investigations involving the urinary metabolites (13, 18, 22). However, Zehnder, *et al.* (23), have, by the use of S^{35}-thioridazine and inverse-isotope dilution techniques, shown that thioridazine disulfone is a metabolite which is found in the bile and urine of the rat. The metabolism of thioridazine is complicated by the presence of another oxidizable sulfur atom in the 2-methylmercapto group of the phenothiazine ring. By inverse dilution analysis, the ring sulfoxide metabolite (the 5-oxide), the side-chain sulfoxide metabolite (oxidation of the methylmercapto sulfur atom), and the disulfoxide metabolite of thioridazine were demonstrated and quantified in addition to the previously mentioned disulfone. The disulfoxide was excreted in the largest quantities. However, all of the metabolites which were determined in the urine represented only 1–2% of the administered radioactivity. These metabolites made up approximately 20% of the radioactive substances in the bile of the rat. Only traces of unchanged thioridazine were excreted in either bile or urine.

Walkenstein and Seifter (24) recovered 70–80% of the radioactivity in the urine and 20–30% in the feces of the dog following a single intraperitoneal injection of S^{35}-promazine. From 3–5% was shown to be the sulfoxide, 2–3% was excreted unchanged, and 3–4% of the dose was comprised of monomethyl promazine and the monomethyl sulfoxide of promazine.

By paper chromatography of urine, Hoffmann, *et al.* (25), have isolated the sulfoxide as a metabolite of mepazine in the rat. Evidence has been given which indicates that a metabolite of perphenazine in liver tissue of the rat is the sulfoxide of this drug (26). Up to one-third of the dose of methoxypromazine is excreted in the urine by man, almost wholly in the sulfoxide form (27). After parenteral administration of promethazine-S^{35} to rats, the largest portion of the radioactive dose appeared in the urine. The sulfoxide of this phenothiazine derivative was found to be an important urinary metabolite in the rat (28). Chlorprothixene [2-chloro-10-(3-dimethylaminopropylidene)thiaxanthene HCl] is subject to biological oxidation in the same manner as the phenothiazine derivatives. A sulfoxide metabolite of this compound has been shown to be formed by man and the rat with the aid of paper chromatography and ultraviolet absorption analysis (29).

The sulfur atom of the phenothiazine ring is particularly subject to oxidation. Apparently the initial stages involve electron transfer and free radical formation. This phenomenon will be discussed later. In studies with mepazine, Block (30) found that sulfoxide formation can occur due to the presence of peroxide contamination of ethyl ether. More recently, Ross, *et al.* (31), have noted *in vitro* sulfoxidation of desmethyl chlorpromazine which occurred during ether extraction or subsequent chromatography. These findings emphasize the care which must be taken in experiments with the phenothiazine compounds in order to prevent (and recognize) the formation of artificial metabolites.

DEMETHYLATION

Ross, *et al.* (32), using chlorpromazine-(N-methyl)-C^{14}, have found that 12–16% of the label is expired as $C^{14}O_2$ by the rat during the first 6 hours. Animals maintained for 12 hours continued to expire 0.7–2.0% of the radioactive dose each hour throughout the observation period. An active system which demethylates drugs has been shown to be present in the liver (33). Upon incubation of chlorpromazine with rabbit-liver homogenates, prepared according to the method of La Du, *et al.* (33), Salzman and Brodie (11) reported that no demethylation occurred. After *in vivo* demethylation of chlorpromazine had been shown (32), Young, *et al.* (34), demonstrated that properly fortified homogenates could demethylate the drug. After a 1-hour incubation, approximately 40% of the substrate carbon-14 could be recovered as liberated CO_2.

The metabolic fate of S^{35}-promazine has been studied in dogs by Walkenstein and Seifter (24). From 70–80% of the administered radioactivity was eventually excreted in the urine. By the use of chromatography and the authentic compounds as non-labeled carriers, several metabolites of promazine were identified: promazine sulfoxide (3–5%); desmethyl promazine sulfoxide (2.5–3.5%); unchanged promazine (2–3%); and desmethyl promazine (0.75–1%). Injection of either desmethyl promazine or promazine sulfoxide gave rise to desmethyl promazine sulfoxide. Thus it appears that neither monodemethylation nor sulfoxidation (as the initial reaction) prevents the second metabolic change resulting in the desmethyl sulfoxide of promazine.

Demethylated derivatives were found to account for at least two of the sulfoxide metabolites noted by Fishman and Goldenberg (13). Additional evidence was given in a later report to substantiate the formation of the desmethyl and desdimethyl sulfoxide metabolites of chlorpromazine (14). The desdimethyl sulfoxide, a primary amine, is suggested to be the major "non-polar" metabolite of chlorpromazine in human urine. Their results may be summarized as follows: 1. Man—desdimethyl chlorpromazine sulfoxide, 3.7%; desmethyl chlorpromazine sulfoxide, 1.8%; chlorpromazine sulfoxide, 0.4%; unchanged chlorpromazine, 0.2%; 2. Dog—desdimethyl chlorpromazine sulfoxide, 1.1%; desmethyl chlorpromazine sulfoxide, 5.1%; chlorpromazine sulfoxide, 5.1%; unchanged chlorpromazine, 2.8%. Huang and Kurland (12) observed the same metabolites by two-dimensional paper chromatography. An additional metabolite of chlorpromazine, believed to be desmethyl chlorpromazine, was noted by Goldenberg and Fishman in the urine of the dog, but not of man. Desmethyl chlorpromazine has been shown to be an *in vitro* metabolite of chlorpromazine (31). Desdimethyl chlorpromazine sulfoxide is a more prominent metabolite of chlorpromazine in man than in the dog. It is possible, considering the fact that the dimethylaminopropyl side chain is common to both promazine and chlorpromazine, that a desdimethyl metabolite of promazine could be demonstrated in man.

With the exception of the unchanged drug in the urine, the excretion patterns are very similar in the rat (18) and the dog (14) after one dose of chlorpromazine. The rat excretes as much as 12% of the dose of chlorpromazine in the urine as unmetabolized drug. Fedorov (17) has reported that urinary excretion of unchanged chlorpromazine by the rat accounts for 25% of the dose given.

The N-demethylation of thioridazine was studied by using the $\diagdown N—C^{14}H_3$ form of the drug (23). Measurement of $C^{14}O_2$ excretion in the respired air indicated that the rat demethylates 30–40% of a dose of this drug. In light of the extensive demethylation of this compound, it is likely that desmethyl analogs of the sulfoxide and sulfone metabolites are also formed. The number of theoretical metabolites is formidable.

Imipramine [N-(3-dimethylaminopropyl)-imino bibenzyl HCl], a compound similar in structure to promazine, is demethylated and hydroxylated. Herrmann, *et al.* (35, 36), found both monomethyl and completely demethylated derivatives of imipramine in rabbit urine. The metabolism of this compound in rabbits is extensive, whereas man excretes much of the ingested imipramine in unmetabolized form.

N-OXIDE FORMATION

As part of their continuing studies on the metabolism of chlorpromazine by man, Fishman, *et al.* (37), have recently reported the isolation of an N-oxide metabolite from urine. The presence of an oxygen atom attached to the nitrogen of the side chain was found to cause minor but detectable differences in the chemical properties of chlorpromazine. It should be noted that the ultraviolet spectra of chlorpromazine and chlorpromazine-N-oxide were indistinguishable. Chlorpromazine-N-oxide was not a major urinary excretion product of chlorpromazine in man or in the dog. In patients receiving chlorpromazine orally, the N-oxide accounted for

0.7% of the daily dose. The corresponding figure for dogs was somewhat higher, as much as 2–3.5%. This work has been extended (38) to include imipramine, which has the same side chain as chlorpromazine. Patients on imipramine therapy excreted approximately 2% of the administered drug as imipramine-N-oxide.

HYDROXYLATION

Upon fractionation of urine from patients receiving chlorpromazine, three polar metabolites were observed by Lin, *et al.* (39). Treatment with β-glucuronidase gave rise to free glucuronic acid and three new phenothiazines. These compounds were not chlorpromazine or chlorpromazine sulfoxide. A fourth metabolite showed an ultraviolet spectrum similar to that of chlorpromazine with an additional absorption peak at 284 mμ, a property suggestive of a ring-hydroxyl function. Following enzymatic hydrolysis, the released phenothiazines became extractable with ethyl ether from an acid medium. This group believes that the products isolated were phenolic metabolites and that hydroxylation constitutes an important aspect of chlorpromazine metabolism in man. Posner (40) has drawn the same conclusion from his chromatographic studies. He had proposed, as have others (2, 12, 14), that glucuronides are the major excretory products in man rather than sulfoxides.

In a study comparing the metabolism of chlorpromazine in man and dog, Goldenberg and Fishman (14) noted both qualitative and quantitative differences in the urinary metabolic patterns. Man excreted predominantly phenolic metabolites of chlorpromazine, and the urine was found to contain one series of phenolic metabolites which were entirely absent in dog urine. These workers estimate that there may be as many as 24 different metabolites of chlorpromazine. Huang and Kurland (2, 12, 41) also reported that hydroxylated products and their glucuronides constitute the chief metabolites of chlorpromazine in man. The glucuronides could be detected for several weeks after discontinuation of treatment.

It was concluded from studies involving β-glucuronidase and paper chromatography that thioridazine is excreted into rat bile primarily as hydroxylated metabolites conjugated with glucuronic acid (23). Thioridazine and its products are excreted primarily in the feces. Studies by Herrmann, *et al.* (35, 36), have shown conclusively that ring hydroxylation occurs in the metabolism of imipramine. The 2-hydroxylated derivative of imipramine has been synthesized and shown to be identical with one of the metabolites in rabbit urine. This metabolite is also excreted as a conjugate with glucuronic acid.

Nadeau and Sobolewski (42, 43) have investigated the excretion products in daily urine samples from patients receiving chlorpromazine over a period of many months. More than half of the compounds excreted in the urine were shown to be present as conjugates, apparently involving glucuronic acid. It was found that the capacity for this conjugation was not altered by massive dosage or prolonged administration of the drug.

In a discussion of the absorption spectra of some metabolites of chlorpromazine, Kinbergen (44) postulated that a certain shift in absorption maxima pointed to the presence of one or two hydroxyl groups on the phenothiazine nucleus. References to conjugated, polar, or possible phenolic metabolites of the phenothiazine derivatives are common (13, 14, 23, 45–47).

Interest in possible hydroxylated metabolites of the amine substituted phenothiazines has been spurred by the knowledge that mono- and di-hydroxylated derivatives of phenothiazine are formed by a number of animals (48–50). The hydroxylated metabolites of phenothiazine are of two basic types—leucophenothiazone (3-hydroxy phenothiazine) and leucothionol (3,7-dihydroxyphenothiazine)—each of which also occurs in an enolic form, phenothiazone (phenothiaz-3-one) and thionol (7-hydroxyphenothiaz-3-one), respectively. Berti and Cima (51) have stated that chlorpromazine compounds of the phenothiazone type are rather improbable. They feel that the substituted nitrogen in chlorpromazine would prevent the shift in double bonds necessary for phenothiazone formation. They also refer to the absence of color in the urine of animals after chlorpromazine treatment. The phenothiazone metabolites impart a red color to the urine of animals treated with phenothiazine. This does not exclude, however, the possibility that certain metabolites of the substituted phenothiazines may be in the leuco or colorless forms analogous to those found with phenothiazine.

OTHER METABOLIC CHANGES

A ferric chloride-sulfuric acid reagent has been developed to yield a color with the metabolites in the urine of a patient which is proportional in intensity to the amount of chlorpromazine ingested daily (52). It was hypothesized that the violet color observed was due to the reaction of

the reagent with a relatively unstable metabolite which is intermediary between chlorpromazine and chlorpromazine sulfoxide. A compound that gives the same violet color can be prepared by irradiation of an aqueous solution of chlorpromazine with ultraviolet light (53). Forrest, *et al.* (53), using electron spin resonance and absorption spectra, tentatively characterized the intermediate as a thionium hydroxide with one or two hydroxyl groups attached to the sulfur atom. The first products in the oxidation of phenothiazine drugs have been shown to be ion radicals (54). There is good evidence that these radicals are responsible for the color reactions observed. The intermediate form of chlorpromazine produced by ultraviolet irradiation is apparently the same as that of the metabolite(s) occurring in the urine of treated patients. It is of interest that small amounts of these compounds are excreted for many weeks following discontinuation of treatment with a phenothiazine drug (3). A free-radical intermediary form of the phenothiazines has also been postulated by Laborit, *et al.* (55). There are several other papers demonstrating the lability of chlorpromazine in the presence of acids, bases, or light (56–59). One analytical method, designed for phenothiazine derivatives, has an end point governed by color changes during titration. A red free-radical is formed immediately upon the loss of one electron. Decoloration occurs at the equivalence point when a second electron is lost (60).

Both the chlorine atom and the side chain of chlorpromazine remain firmly attached to the phenothiazine nucleus during the passage of the drug through the animal body (51). The stability of the halogen atoms of trifluoperazine has also been demonstrated (61).

There is evidence that the thiazine ring can be degraded by the mouse. Christensen and Wase (15) recovered small quantities of the administered radioactivity as labeled sulfate after injection of S^{35}-chlorpromazine. Walkenstein and Seifter (24) have reported that 10% of the sulfur from S^{35}-phenothiazine is excreted by the dog as inorganic sulfate. This type of breakdown did not occur with promazine, however. This provides further evidence that the side chain is not metabolically removed.

Acetylfluphenazine can be enzymatically hydrolyzed *in vitro* to fluphenazine. Smith (62) has hypothesized that the same reaction may take place *in vivo* and that fluphenazine is the active agent.

The biological fate of two quaternary phenothiazine compounds has been studied in rats by the use of sulfur-35 and tracer techniques (28, 63). After oral administration, these compounds are excreted mainly in the feces. One was largely excreted as the unchanged drug with only traces of possible metabolites. Although the sulfoxide was observed with the other compound, only a small portion of the dose appeared in this form. Apparently neither compound undergoes extensive metabolism. Access to the metabolizing enzymes may be prevented by the chemical influence of the quaternary nitrogen of the side chain in these compounds.

PHARMACOLOGICAL EFFECTS OF METABOLITES

Although chlorpromazine sulfoxide exhibits pharmacological effects which are qualitatively similar to those of chlorpromazine, the latter compound has a much greater potency and a more rapid onset of action (64). Chlorpromazine sulfoxide has been shown to have a weak sedative action of short duration in man (21). For these reasons it is considered unlikely that the effects of chlorpromazine are mediated through the sulfoxide. Moran and Butler (64) have shown that chlorpromazine sulfoxide is not equally active in all species. The sulfoxide was found to be about one-tenth as active as chlorpromazine in potentiating hexobarbital anesthesia and one-eighth as active as chlorpromazine in producing sedation in the dog. Cats exhibited similar effects. In mice and rabbits, however, chlorpromazine sulfoxide was almost devoid of sedative action and had very little potentiating effect on hexobarbital sleeping time in mice. This is in marked contrast to chlorpromazine which is about equally potent, in these respects, in all of the forenamed species. The pharmacological activity of mepazine shows a decrease corresponding to that seen with chlorpromazine upon sulfoxidation (25).

Posner, *et al.* (4), have compared, in a series of behavioral and pharmacological tests, several known and model metabolites of chlorpromazine and promazine. Their results confirmed those of previous reports concerning the decreased activity of chlorpromazine sulfoxide. Of greatest interest, however, was the remarkable degree of activity shown by demethylated and hydroxylated compounds. Desmethyl chlorpromazine was only slightly less active than chlorpromazine. While the activity of chlorpromazine-N-oxide was less than desmethyl chlorpromazine, all of these chlorpromazine analogs were far more active than chlorpromazine sulfoxide. The ac-

tivity of 4-hydroxypromazine approached that of promazine, but the 2-hydroxy derivative was generally much less active. Although these phenolic derivatives of promazine are only model compounds and are definitely not metabolites of the drug (40), other phenolic metabolites are known to be formed. The studies on model compounds clearly demonstrate that activity could reside in a metabolite resulting from ring hydroxylation. The possibility that demethylated or N-oxide metabolites contribute to the action of chlorpromazine or promazine is equally intriguing.

In chemical studies chlorpromazine was found to be a powerful electron donor. This property was attributed to the phenothiazine portion of the molecule. Karreman, *et al.* (65), have suggested that the therapeutic effect of the drug is related to this phenomenon.

Investigators have shown that desmethylimipramine, a known metabolite of imipramine is more potent in some respects than its parent compound (66). There is good evidence to indicate that the antidepressant action of imipramine is actually due to its demethylated metabolite (67, 68). It is more remarkable, however, that imipramine appears to antagonize the effectiveness and delay the onset of action of desmethylimipramine (68). These results serve to emphasize the problems which may also be encountered in studies on the phenothiazine derivatives concerning the evaluation of the pharmacological actions and interactions of parent compound and metabolites.

SUMMARY

Consideration has been given to the literature associated with the metabolism of modern phenothiazine drugs. Several important pathways have been elucidated. A particularly characteristic reaction of this class of compounds is sulfoxidation. Further oxidation of the sulfur atom to a sulfone has been observed only with thioridazine. A number of other metabolic reactions have been demonstrated with the phenothiazine drugs. Among these are: demethylation, N-oxide formation, and conjugation with glucuronic acid. Although the isolation and identification of an actual phenolic metabolite of a N-substituted phenothiazine has not yet been accomplished, the analysis of glucuronic acid conjugates leaves little doubt that the phenothiazine components are phenolic in nature. Evidence indicates that aromatic hydroxylation followed by conjugation with glucuronic acid is the dominant metabolic pathway for the phenothiazine drugs in man. Studies have shown that it is possible for a phenolic metabolite to contribute to the action of its parent drug, *e.g.*, aromatic hydroxylation does not necessarily result in loss of pharmacological activity. Thus, the identification of the phenolic metabolites of the phenothiazine derivatives should be considered a task of prime importance.

In view of the number of known and theoretical metabolites of the phenothiazine drugs, the species differences in the metabolism of these compounds, and the inherent lability of the phenothiazine nucleus, one must conclude, in retrospect, that the progress in elucidating the metabolic fate of these compounds has been quite remarkable. However, much work remains to be done in this area, since with the metabolites identified to date it is not possible to account for more than 8–10% of the dose of a phenothiazine drug in man.

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Research Articles

Quantitative Determination of Serotonin in *Panaeolus* Species

By J. K. WIER† and V. E. TYLER, JR.

A method was developed for the quantitative estimation of serotonin (5-hydroxytryptamine) in mushrooms, especially species of the genus *Panaeolus*. The procedure involves extraction of the dried, finely powdered carpophores with acidified alcohol, application of the extract to filter paper strips, formation of these chromatograms with *n*-propanol : 1 *N* ammonium hydroxide (5:1), development of colored serotonin spots with a modified Pauly's reagent, determination of the total color of these spots with an automatic recording electronic densitometer, and calculation of the serotonin content from a standard curve. Each step in the procedure was evaluated to determine the influence of certain variables, and the results obtained by the final method were subjected to statistical evaluation.

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Serotonin and related indole derivatives have been quantitated by determination of their physiological effects on certain animal tissues,

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such as isolated intestinal strips (6), and on grass coleoptiles (7). Other methods involving absorption of ultraviolet light, fluorescence, and colorimetry (8) have also been utilized. Difficulties involved in separating serotonin from the closely related compounds with which it frequently occurs, render all existing procedures somewhat tedious to employ and more or less nonspecific. These considerations prompted us to undertake the development of a new quantitative procedure for the determination of serotonin in mushroom tissues. Consequently, a quantitative paper chromatographic assay procedure was designed and critically evaluated.

The general procedure followed in the estimation of serotonin was to extract the serotonin from dried carpophores of *Panaeolus* species by suitable means, to spot these extracts and known serotonin solutions on filter paper strips, to prepare chromatograms on these strips with suitable developing solvents and color-forming reagents, to determine the total color of the serotonin chromatographic spots with an automatic recording electronic densitometer, and to estimate the serotonin content of the mushrooms by comparison of the total color determinations of the extract spots with those produced by known quantities of serotonin.

During the course of this investigation, a preliminary communication appeared which called attention to the possibility of quantitation of serotonin and other 5-hydroxyindoles by similar paper chromatographic methods (9). This publication did not give experimental details regarding the influence of the numerous variables involved, nor did it apply the method to extracts of natural products. Preliminary experimental evidence available at the time this work came to our attention revealed that the accurate paper chromatographic determination of serotonin was by no means as simple as the publication of Carcasona, Unterharnscheidt, and Cervos-Navarro would seem to indicate. This fact was supported by continued studies.

EXPERIMENTAL

The mushrooms utilized in this investigation were specimens of the dried carpophores of *Panaeolus campanulatus* (Fr.) Quél. and *P. foenesecii* (Fr.) Kühn. which were collected in the Puget Sound region of the state of Washington. Samples of both species were authenticated by Prof. Alexander H. Smith, University Herbarium, University Museums, University of Michigan. Since fresh carpophores could not be preserved for extended periods of time, they were dried for 48 hours in a forced-air drying oven at approximately 45°. This procedure resulted in an average loss in weight (water) corresponding to 93.5% of the fresh tissue.

Serotonin creatinine sulfate[1] (California Corporation for Biochemical Research) was used as a reference standard. Standard solutions of this serotonin complex were prepared in 0.1 *N* hydrochloric acid in diluted alcohol, U.S.P. XVI, and were preserved at room temperature in the dark. Under these conditions, the solutions were found to be stable for several weeks.

Extracts of mushroom carpophores were prepared by homogenizing for 20 minutes an accurately weighed quantity (approximately 1 Gm.) of a No. 200 powder of the tissues in about 7–8 ml. of 0.1 *N* hydrochloric acid in diluted alcohol, U.S.P. XVI, by means of a glass homogenizing tube fitted with a Teflon pestle. The particulate matter was separated by centrifugation. Portions of the supernatant liquid, representing the extract of a known tissue weight, were applied by means of a micropipet to 1-in. wide strips of Whatman No. 1 filter paper (designated for chromatographic use). The solutions were applied in 2–3 μl. portions, with drying between applications, to a maximum volume of 50 μl. It was found that while serotonin in the standard solutions was not affected by hot air drying, approximately 25% of the serotonin in the homogenate supernatant liquid was destroyed when the temperature of the drying air was approximately 65–70° (Table I). Consequently, forced air at room temperature was used for drying the spotted solutions.

TABLE I.—EFFECT OF HOT AIR DRYING ON PURE SEROTONIN COMPLEX AND SEROTONIN IN MUSHROOM EXTRACT

Compound on Strips	Blower Temperature	No. of Strips	Mean Serotonin Spot Size, in.²	95% Confidence Limits, in.²
Serotonin complex	cold	10	3.96	±0.116
Serotonin complex	hot	10	3.93	±0.195
Mushroom extract	cold	8	2.02	±0.079
Mushroom extract	hot	8	1.54	±0.053

Chromatograms were formed by the ascending method employing solvent systems of either the butanol phase of *n*-butanol : glacial acetic acid: water (4:1:5) or *n*-propanol : 1 *N* ammonium hydroxide (5:1). After drying for 2 hours at room temperature, the formed chromatograms were sprayed with either *p*-dimethylaminobenzaldehyde (2%) in hydrochloric acid : water (1:9), or with a diazotized sulfanilamide solution followed by an alkaline spray (Pauly's reagent) (10). In this investigation, 1 *N* ammonium hydroxide was substituted for the sodium carbonate solution usually employed in Pauly's reaction because carbonate left a residue on the paper strips which made the rate of feed of those strips through the scanning apparatus very uncertain. Chromatographic strips were sprayed while in a horizontal position to minimize the eluting effect of the spray solutions.

[1] A quantity of the compound equal to that purchased was supplied through the courtesy of Vismara Terapeutica, Casatenova brianza (Como), Italy.

The color formed by serotonin and the Pauly-ammonium hydroxide reagent was extremely stable. No appreciable loss of color could be detected after 6 hours; approximately 5% of the color intensity was lost after one week. The color formed with the *p*-dimethylaminobenzaldehyde reagent was very unstable; approximately 11% of this color was lost after only 2 hours.

Total color of the serotonin-reagent spots was determined with a Photovolt automatic recording electronic densitometer. This apparatus consisted of the following: (*a*) photometer, model 501-A with phototube C and a 485 mμ (Pauly reaction spots) narrow-band color filter; (*b*) transmission density unit, model 52C, with a 1 × 25 mm. aperture slit, a guide for the paper strips, and a synchronous motor drive assembly to advance the paper strips; (*c*) varicord, variable response recorder model 42. This apparatus moved the strips past the 1 × 25 mm. aperture which was located between the light source and the photoelectric cell. The chart paper of the recorder was moved in synchronization with the movement of the chromatographic strip past the aperture. The excursion of the pen on the recorder chart paper was proportional to the total absorption of light across the width of the paper strip. The area under the generally triangular-shaped curves traced by the recorder was a measure of the total color of the chromatographic spots scanned. This area was measured with a polar planimeter.

Because the density of the filter paper strips along their length was quite variable, it was necessary, for greatest precision, to apply a correction for this variation to the recorder chart base line. This was prepared by passing the blank strips through the densitometric apparatus before solutions were spotted on them and using the recording thus obtained as a base line for the curves produced from the finished chromatograms. A typical series of ten strips examined in this way, and the total spot size calculated using the variable base line, gave a mean result (95% confidence limits) of 5.35 ± 0.180 in.² in comparison to a mean of 5.20 ± 0.333 in.² when a straight base line was employed.

Standard curves were prepared for serotonin (standard solutions of serotonin creatinine sulfate) in both the *n*-butanol : glacial acetic acid : water and the *n*-propanol : 1 *N* ammonium hydroxide solvent systems, using the Pauly-ammonium hydroxide spray reagent. Table II and Fig. 1 present the data for the latter solvent system.

TABLE II.—TOTAL COLOR DETERMINATIONS OF SEROTONIN EMPLOYING *n*-PROPANOL : 1 *N* AMMONIUM HYDROXIDE CHROMATOGRAPHIC SYSTEM AND MODIFIED PAULY SPRAY

Serotonin, mcg.	No. of Strips	Mean Area, in.²	95% Confidence Limits, in.²
1	18	0.81	±0.046
2.5	18	1.98	±0.077
4	18	3.04	±0.058
5	18	3.59	±0.061
7.5	12	4.61	±0.155

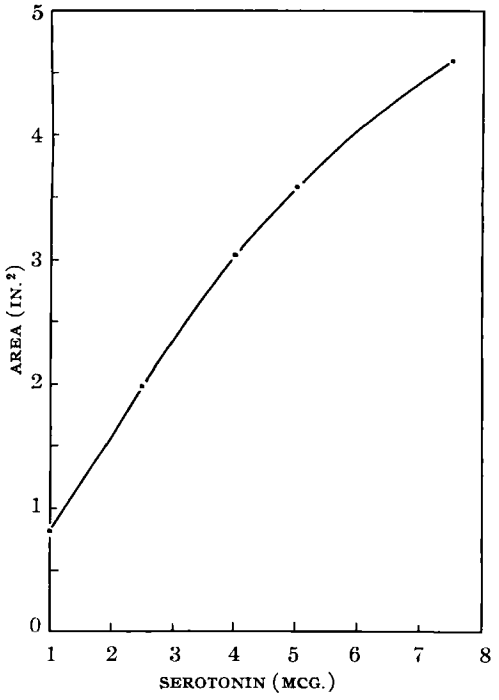

Fig. 1.—Standard curve prepared from total color determinations of serotonin spots following chromatography in *n*-propanol : 1 *N* ammonium hydroxide system and treatment with modified Pauly spray.

RESULTS AND DISCUSSION

As seen in Fig. 1, the relation of total color of chromatographic spots to the concentration of serotonin in those spots is not linear, nor does a simple logarithmic relationship exist between these two quantities. This is not an unexpected result, since the effect of the paper strip, the nature of the color-forming reaction, and the quantities of substance required in the spots for satisfactory determination of total area may all act to cause such a nonlinear relationship. The slight curvature of this line imposes no impediment to reliable assay determinations.

In order to determine the efficiency of the extraction procedure and the accuracy of the total color densitometric assay method, known amounts of serotonin were added to carpophore tissue, extracted, and quantitated by both ultraviolet light absorption and the chromatographic procedure. In addition, serotonin content of carpophore tissue was determined by a nitrosonaphthol colorimetric assay (8).

The ultraviolet absorption technique revealed approximately 95% recovery of added serotonin; the total color densitometric procedure gave a similar value for chromatograms formed in the *n*-propanol:1 *N* ammonium hydroxide solvent system. However, this procedure when applied to chromatograms formed in the *n*-butanol:glacial acetic acid:water solvent system, showed approximately only 62% recovery of added serotonin; and the value of mushroom tissue serotonin determined from such chromatograms was approximately 62% of that indicated by chromatograms formed in the *n*-propanol:1 *N* ammonium hydroxide solvent system. Since the only difference between the two cases was in the solvent systems employed, this apparent difference in mushroom tissue serotonin

content must have been due to a difference in interaction between those two solvent systems with the substances present in the mushroom extract.

Tyler and Smith (5) have shown, by the use of two-dimensional chromatographic procedures applied to extracts of *P. campanulatus*, that the serotonin spot formed in the *n*-propanol:1 *N* ammonium hydroxide solvent system is not separable into other Pauly reagent-positive spots. Therefore, the low concentration of serotonin indicated by the chromatograms formed in the *n*-butanol:glacial acetic acid:water solvent system must have been due to a failure of a portion of the serotonin present in the extracts to migrate to the normal serotonin position on the chromatograms. As Stowe has suggested (11), such failure could be due to the existence of serotonin as more than one ionic species in the presence of this solvent system and other substances present in the extracts.

Serotonin content of mushroom tissues estimated by the total color densitometric system from chromatograms formed in the *n*-propanol:1 *N* ammonium hydroxide solvent system agreed reasonably well with that determined by the nitrosonaphthol colorimetric procedure, but consistently averaged approximately 10% higher. This would seem to indicate a lower efficiency of serotonin extraction by the procedure used for the nitrosonaphthol determination. Davis (12) has indicated that this procedure gives a low value of blood serotonin due to oxidation occurring during the extraction process.

The mean serotonin content of five samples of *P. campanulatus* determined by the total color procedure presented here was 1234 ± 39.0 mcg./Gm. (Table III). Correction for the mean water content

TABLE III.—SEROTONIN CONTENT OF DRIED CARPOPHORES OF *Panaeolus campanulatus*[a]

Sample No.	Serotonin Content, mcg./Gm.
1	1211
2	1266
3	1240
4	1193
5	1260
Mean[b]	1234 ± 39.0

[a] Extracted by homogenization in acidic diluted alcohol, U.S.P. XVI. Assayed by total color method using *n*-propanol:1 *N* ammonium hydroxide (5:1) solvent system and modified Pauly spray reagent. [b] Mean ±95% confidence limits.

gives a value of 80 mcg. of serotonin per gram of fresh mushroom tissue (0.008%). The serotonin content of *P. foenesecii*, similarly established, was 3728 ± 45.8 mcg./Gm. (dry weight) or 240 mcg./Gm. of fresh tissue (0.024%). Only one plant material, the kernel of the English walnut, *Juglans regia*, has been reported to have a higher serotonin content (170–340 mcg./Gm.–fresh weight) (13).

SUMMARY

A summary of the total color densitometric assay procedure is as follows:

1. Homogenization of a No. 200 powder of dried mushroom carpophores with 0.1 *N* hydrochloric acid in diluted alcohol, U.S.P. XVI.

2. Quantitative application of these carpophore extracts to filter paper strips with forced air drying of the spots at room temperature.

3. Formation of chromatograms by the ascending method employing an *n*-propanol:1 *N* ammonium hydroxide (5:1) solvent system.

4. Formation of colored serotonin spots with Pauly-ammonium hydroxide reagent.

5. Determination of total color of serotonin spots with an automatic recording electronic densitometer.

6. Calculation of mushroom serotonin from a standard curve prepared by chromatographing known quantities of serotonin creatinine sulfate.

The extraction procedure outlined here is rapid and simple. Although the determination of total color of the chromatographic spots is time consuming, the extreme stability of the serotonin-Pauly reagent color lends itself well to such a technique. Inspection of the 95% confidence limit values presented in Tables II and III shows that the precision of the method is quite acceptable, rendering it a useful procedure for the specific determination of serotonin in plant tissues.

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Investigation of the Cardiovascular Response of the Dog to 1-Phenyl-2-hydrazinopropane

By STUART BOYER, HOWARD J. JENKINS, and SUMNER M. ROBINSON

Results of a further attempt to determine a basis for the qualitative difference in blood pressure response of the human and the dog to 1-phenyl-2-hydrazinopropane are reported. Elimination of arterial pressure level as a factor influencing the circulatory response of the dog to this compound removes the last possibility for a circumstantial basis for the difference. Mechanism studies indicate that the canine pressor response is a neurotropic, sympathetic effect mediated, for the most part, as a direct action on myoneural junction receptor sites with a lesser component of indirect action on these sites related to MAO inhibition or the peripheral release of catecholamines from blood vessel walls. Evidence presented by certain clinical investigators points to either a ganglionic or an adrenergic block as responsible for the human antihypertensive effect.

WHILE 1-phenyl-2-hydrazinopropane[1] has been reported to produce marked orthostatic hypotension in humans with elevated blood pressure, it would appear to elicit only an inconstant and relatively weak depressor response in the supine hypertensive (1, 2). Certain clinical studies have indicated that this hydrazine analog of amphetamine exhibits only a slight hypotensor activity in normotensive individuals. These findings are in sharp disagreement with the pressor activity demonstrated in both pentobarbital-anesthetized and conscious dogs by Groves (3) and in the present work with conscious dogs in the orthostatic position.

The goal of this and a previous investigation was the establishment of a basis for the apparent species difference in the responses of the human and the dog to 1-phenyl-2-hydrazinopropane. Realization of this goal was attempted first through the stepwise elimination of species differences pertaining to the conditions surrounding the administration of the drug on the one hand, and the measurement of blood pressures on the other. A second attempt at goal achievement involved a study of the mechanism of the hypertensive response of the dog to this compound. Information provided in the latter study together with that relating to the mechanism of the hypotensive response of the human to this drug might provide the explanation for a true species difference in response.

EXPERIMENTAL

Methods.—Since the work of Groves served to eliminate route of administration, anesthesia and recording position of the individual as bases for the difference in the response of the human and the dog to 1-phenyl-2-hydrazinopropane, the first concern of this work was to examine the influence of blood pressure level on the canine response to this compound. Accordingly, Page's method was employed for the production of chronic renal hypertension in dogs (4). Work with 1-phenyl-2-hydrazinopropane was begun only after significant sustained elevation in blood pressure was observed, usually about 4 to 5 weeks after the encapsulation of the kidneys.

Blood pressure recordings were obtained in these renal hypertensive animals both in the conscious and pentobarbital-anesthetized state by means of a femoral artery puncture. In conscious dogs the puncture area was previously anesthetized with 2% ethyl chloride. A continuous flow of a 0.02% heparin solution in saline (5 ml./hour) from the infusion pump prevented clotting in the artery. The infusion pump was connected to a Sanborn recorder through a transducer and amplifier. Changes in respiratory rate and depth were recorded by means of a bellows-type pneumograph which was connected through another transducer and another amplifier to the same recorder.

All injections of this compound were made in the femoral vein through an 18-gauge indwelling needle which was connected by rubber tubing to a saline-filled buret. The injection volume was flushed in each time at the same rate with the same saline volume.

Results.—Prior to the actual testing of the compound in hypertensive dogs, work was carried out in normotensive animals, both conscious and pentobarbital-anesthetized (see Table I). The data obtained were qualitatively similar to but quantitatively different from comparable results appearing in the literature. The cardiac rate decrease generally took place in normotensive dogs at the beginning of blood pressure elevation rather than at or after the peak response was reached. In a few instances, a cardiac rate increase rather than a decrease followed administration of the compound, and when this occurred the pressor response was more marked than when a decrease in rate resulted. Work with atropinized dogs indicated that the cardiac rate decrease usually seen in normotensive dogs is reflex-ordered.

The peak blood pressure response in both con-

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[1] Supplied by Lakeside Laboratories.

TABLE I.—BLOOD PRESSURE EFFECTS OF 1-PHENYL-2-HYDRAZINOPROPANE IN THE NORMOTENSIVE DOG

Dog No.	Dose, i.v., mg./Kg.	No. Expt.	Av. Mean Pressure	Av. Rise Mean Pressure
		Anesthetized		
1	0.25	3	162	52
	0.50	3	161	66
	0.75	3	159	68
	1.0	2	152	96
2	0.50	2	147	92
	0.75	2	143	104
	1.0	2	160	119
		Conscious		
1	1.0	4	167	117
2	1.0	4	164	116
		Anesthetized-Phentolamine Pretreated		
1	1.0	3	135	7

scious and anesthetized normotensive dogs was attained, in every case, within $^1/_2$ to $2^1/_2$ minutes subsequent to administration of the compound. The respiratory rate was usually increased when initially low and decreased when initially high. In most instances, the depth of respiration was decreased when blood pressure reached peak response, but the effect was of short duration only.

A pressor activity, corresponding in extent and duration to that evidenced in normotensive dogs, was exhibited by 1-phenyl-2-hydrazinopropane in hypertensive dogs (see Table II). Elevation of blood pressure usually began immediately after administration of the compound to either normotensive or hypertensive dogs. In most instances a decrease in cardiac rate, beginning with blood pressure rise and continuing until return of pressure to preinjection levels, occurred in both animals. Peak pressures were reached in hypertensive dogs within the same time interval as in normotensive dogs. Respiratory changes were similar in both animals, the rate being increased if previously low and decreased if previously high, and depth being decreased fleetingly during the zenith of the blood pressure response.

Tolerance to the compound was reflected in the tachyphylaxis associated with pressor responses to repeated doses in a given test run. Also a distinct sedating effect, related in magnitude to dose, accompanied other manifestations of activity. The latter effect had a duration similar to that of the blood pressure response. Salivation occurred intermittently, and tachycardia and mydriasis were occasionally encountered following the administration of 1-phenyl-2-hydrazinopropane. Ataxia was noted in hypertensive dogs after two injections of 2.0 mg./Kg. Cardiac arrhythmias usually became more marked as the dosage level was increased. Soft stools and emesis were observed after repeated administration of the compound.

With the elimination in the portion of the work of the influence of pre-existing blood pressure as a factor in the response to 1-phenyl-2-hydrazinopropane in the dog, the last of the circumstantial bases for an apparent species difference in the response of the human and the dog to this compound was removed.

Since the mode of action involved in the pressor response to 1-phenyl-2-hydrazinopropane in the dog is not generally agreed upon by investigators, it was considered advisable to pursue this area further in an effort to uncover a mechanistic basis for the compound's species response difference. If there is a definitive difference in the pharmacology of this compound at the action level in the human and in the dog, as indicated by this and comparable clinical studies, this would, of course, account for the response difference in the two species. A number of dogs were used in this portion of the investigation, but in a given series, to avoid animal variation, the same dog was employed repeatedly.

Results of experiments by Eltherington and Horita in intact dogs demonstrate the ability of dibenzyline to block completely the pressor response to 1-phenyl-2-hydrazinopropane (5). These findings support the likelihood of a neurotropic, rather than a musculotropic, activity in the dog's pressor response to this compound. That this neurotropic activity is not concerned with a block of parasympathetic pathways is supported by other findings of these investigators in intact dogs. Previous administration of the compound to such animals was shown to cause no significant changes in the pressure responses elicited by acetylcholine chloride (10 mcg./Kg.).

The possibility that the neurotropic activity is an active proposition involving sympathetic pathways, either the ganglia or the myoneural junction, was contemplated. The appraisal of this possibility comprises the latter portion of this investigation. The postulation of ganglionic stimulation as the mechanism of action of 1-phenyl-2-hydrazinopropane was examined with the use of the ganglionic blocking agent hexamethonium. The latter was administered by intravenous injection (2 mg./Kg.) to anesthetized normotensive and conscious hypertensive dogs prior to the administration of 1 and 2 mg./Kg. doses of 1-phenyl-2-hydrazinopropane by the same route. Hexamethonium did not exert any observable effect on the pressor response of either animal to 1-phenyl-2-hydrazinopropane. Actually, the reverse was true, *i.e.*, following the

TABLE II.—BLOOD PRESSURE EFFECTS OF 1-PHENYL-2-HYDRAZINOPROPANE IN THE HYPERTENSIVE DOG

Dog No.	Dose, i.v., mg./Kg.	No. Expt.	Av. Mean Pressure	Av. Rise Mean Pressure
		Anesthetized		
3	0.1	4	174	45
	0.25	7	183	96
	0.50	4	186	114
	0.75	2	169	140
	1.0	4	172	155
4	0.25	2	177	54
	0.50	2	179	98
	0.75	3	180	103
	1.0	3	178	115
		Conscious		
3	0.1	2	197	30
	0.25	3	198	51
	0.50	5	200	77
	0.75	7	201	85
	1.0	3	186	108
4	0.50	3	198	68
	0.75	3	174	70
	1.0	3	174	68

former, the pressor response to the latter appeared enhanced. The effectiveness of the hexamethonium block was confirmed by the subsequent injection of 1-dimethyl-4-phenylpiperazine iodide (DMPP).

With ganglion stimulation thus ruled out of sympathetic involvement, a next step was to test the postulation of adrenergic receptor site stimulation. This was done by administering 25 mg./Kg. of phentolamine[2] (methanesulfonate) intravenously prior to the compound to block these receptor sites at the sympathetic myoneural junction. The administration of 1-phenyl-2-hydrazinopropane in amounts which, in the absence of phentolamine, had been effective in eliciting a marked elevation of blood pressure, failed to evoke a significant pressor response (see Table I). Confirmation of the adrenergic blockade was provided by a fall in blood pressure following intravenous administration of 4 mg. of epinephrine hydrochloride. The intravenous administration of RA-1226,[3] a compound which has been demonstrated to possess both neurotropic and musculotropic spasmogenic activity (6), was found effective in elevating canine blood pressure following doses of 1 to 25 mg./Kg. of phentolamine. This latter finding corroborates the discovery by Eltherington and Horita of the lack of a musculotropic spasmogenic component in the pharmacologic makeup of 1-phenyl-2-hydrazinopropane.

Since the previous work had thus limited the action site of the pressor response to 1-phenyl-2-hydrazinopropane in the dog to the sympathetic myoneural junction, a distinction had to be drawn among the three conceivable types of action at this point: direct stimulation of junctional receptor sites, indirect stimulation of these sites through peripheral release of catecholamines stored in the blood vessel walls (7) and monoamine oxidase (or similar enzyme) inhibition (8). It was thought that one or a combination of these types actually occurs. In an effort to determine which, 1-phenyl-2-hydrazinopropane was administered to normotensive dogs previously reserpinized (chemical sympathectomy) by the subcutaneous injection of 0.05 mg./Kg. for 5 to 7 consecutive days (see Table III). The pressor response to the compound in such animals was not so great by some 40% as in the nonreserpinized dogs, these results indicating that the major part of the compound's pressor activity is exerted by direct stimulation of adrenergic receptor sites, the remainder through either MAO inhibition or the peripheral release of catecholamines stored in blood vessel walls. Studies conducted in dogs pretreated with 15 mg./Kg. of guanethidine for periods of 24 to 48 hours prior to the administration of 1-phenyl-2-hydrazinopropane support the above results.

TABLE III.—BLOOD PRESSURE EFFECTS OF 1-PHENYL-2-HYDRAZINOPROPANE IN THE RESERPINIZED DOG

Dog No.	Dose, i.v., mg./Kg.	No. Expt.	Av. Mean Pressure	Av. Rise Mean Pressure
		Anesthetized		
1	0.25	2	118	30
	0.50	2	105	33
	0.75	3	127	43
	1.0	2	123	70
		Conscious		
2	1.0	4	146	72

DISCUSSION

The discovery in the first portion of this work that the arterial blood pressure level of the dog receiving 1-phenyl-2-hydrazinopropane does not materially affect his blood pressure response to this compound served to remove the last possibility for a circumstantial basis for the difference in its effect upon human and canine circulatory systems. There remained then the task of uncovering, from a mode of action study, a possible mechanistic basis for the species response difference.

The data of Eltherington and Horita demonstrating the ability of dibenzyline to block effectively the pressor response to this compound in the anesthetized dog point to the lack of a musculotropic component in its activity makeup. Support for the lack of musculotropic activity on the part of 1-phenyl-2-hydrazinopropane is provided by that portion of this study in which phentolamine (25 mg./Kg.) was shown capable of preventing the effect of this compound while RA-1226, an agent possessing both neurotropic and musculotropic activity, was only partially blocked by the same dose of adrenergic blocker. Eltherington and Horita also maintained that the neurotropic action of 1-phenyl-2-hydrazinopropane is not involved with the parasympathetic pathway, since previous administration of the compound caused no alteration in the blood pressure response to acetylcholine. In the current study, it was shown that the previous administration of blocking doses of hexamethonium does not alter the ability of the compound to elevate pressure. From this information, it would appear that stimulation along sympathetic pathways does not occur at the ganglia nor is it of central origin. Data of similar investigations with tetraethylammonium by Eltherington and Horita concur in these findings.

The rise in blood pressure following 1 mg./Kg. doses of 1-phenyl-2-hydrazinopropane was completely blocked by phentolamine (1 and 25 mg./Kg.), partially blocked by guanethidine (15 mg./Kg.), and reserpine (0.05 mg./Kg. for 5 days). These results point to four action possibilities with respect to the compound's pressor effect in dogs. These possibilities include direct stimulation of effector cell receptor sites at the sympathetic myoneural junction, indirect stimulation of these sites through MAO inhibition, indirect stimulation through the peripheral release of catecholamines stored in the blood vessel walls and, finally, a combination of two or more of these.

The results in dogs pretreated with reserpine and guanethidine indicate that the major component of the pressor activity of this agent, some 60%, is exerted through direct stimulation of adrenergic receptors. The lesser activity component, the 40% blocked by reserpine and guanethidine, is achieved through either MAO (or other enzyme) inhibition or the peripheral release of catecholamines stored in the walls of the blood vessels. In the human, the compound may act like a ganglionic blocker (9) or an adrenergic blocker (10).

[2] Marketed as Regitine (methanesulfonate) by Ciba Pharmaceutical Co.

[3] 4-Methyl-2-aminopyridine, supplied by Lakeside Laboratories.

SUMMARY

The pressor response to 1-phenyl-2-hydrazinopropane is essentially the same in hypertensive as in normotensive dogs.

The cardiac rate decrease which accompanies the blood pressure elevation following the administration of this compound does not occur in atropinized dogs.

The pressor response to 1-phenyl-2-hydrazinopropane is completely blocked by phentolamine, indicating the absence of a musculotropic component and the involvement of the sympathetic pathway in the response.

The pressor response to this compound is not affected by the prior administration of hexamethonium, making unlikely the possibility of ganglion and central stimulation.

The diminution of the pressor response to 1-phenyl-2-hydrazinopropane in reserpinized dogs and those which had received guanethidine gives support to the combined mechanism at the myoneural junction of direct and indirect effector cell receptor site stimulation, the latter by MAO (or other enzyme) inhibition or by the peripheral release of catecholamines in the blood vessel walls.

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Phase Solubility Study of Solid Species Formed by Magnesium Aluminate from Aqueous Solutions Containing Sulfate Ions

By TAKERU HIGUCHI and FOO SONG HOM†

A phase solubility technique has been used to detect and establish the nature of several hydrated solid species formed by sulfated magnesium aluminate. The most stable form at lower magnesium level appears to correspond to a hydrate of 4MgO : Al_2O_3:SO_3. Equilibrium behavior of this substance is such as to obey the solubility product principle with regards to both magnesium and sulfate. Under other conditions species corresponding to MgO:Al_2O_3, 3MgO : Al_2O_3 : SO_3, and 8MgO:Al_2O_3 : SO_3 seem to be produced. The results demonstrate the great value of the phase solubility technique in studying complex inorganic systems.

SERIOUS INVESTIGATIONS of the physical states of precipitated hydrous oxides and mixed hydrous oxides have been severely limited by the instability of these inorganic systems and by the difficulty in establishing their true states and compositions without washing or drying. For this reason, despite wide pharmaceutical usage of many of these compounds as bases for antacids, no detailed physical-chemical studies on these systems have been published in recent years. The present report is concerned with the results of an investigation on a hydrous sulfated magnesium aluminate system which makes use of a phase solubility technique developed earlier for studies on formations of organic complexes. The data presented show that the $m\text{MgO}\cdot n\text{Al}_2\text{O}_3\cdot p\text{SO}_3\cdot x\text{H}_2\text{O}$ systems could exist in a number of different stoichiometric ratios.

Results of the present studies suggest that formations of distinct solid species having the following approximate compositions are favored by hydrated aluminum and magnesium oxides in contact with aqueous solutions containing sulfate ions: (*a*) $3\text{MgO}\cdot\text{Al}_2\text{O}_3\cdot\text{SO}_3\cdot x\text{H}_2\text{O}$, (*b*) $4\text{MgO}\cdot\text{Al}_2\text{O}_3\cdot\text{SO}_3\cdot y\text{H}_2\text{O}$, and (*c*) $8\text{MgO}\cdot\text{Al}_2\text{O}_3\cdot\text{SO}_3\cdot z\text{H}_2\text{O}$. The presence of sulfate in the structure appears to be necessary. These systems appear to possess distinct solubility products based on the concentrations of the three components.

These findings also attest to the value of phase solubility determinations in the detection and

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SUMMARY

The pressor response to 1-phenyl-2-hydrazinopropane is essentially the same in hypertensive as in normotensive dogs.

The cardiac rate decrease which accompanies the blood pressure elevation following the administration of this compound does not occur in atropinized dogs.

The pressor response to 1-phenyl-2-hydrazinopropane is completely blocked by phentolamine, indicating the absence of a musculotropic component and the involvement of the sympathetic pathway in the response.

The pressor response to this compound is not affected by the prior administration of hexamethonium, making unlikely the possibility of ganglion and central stimulation.

The diminution of the pressor response to 1-phenyl-2-hydrazinopropane in reserpinized dogs and those which had received guanethidine gives support to the combined mechanism at the myoneural junction of direct and indirect effector cell receptor site stimulation, the latter by MAO (or other enzyme) inhibition or by the peripheral release of catecholamines in the blood vessel walls.

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characterization of complex inorganic species. No other technique at the moment can give such unequivocal evidence of formation of these discrete phases.

PAST WORK

Interactions of aluminum and magnesium oxides in more or less their hydrated state to form complex species have received serious attention by numerous previous workers (1–17). In many instances no more was claimed than that an amorphous mixture of improved therapeutic value as an antacid was claimed. In other cases there were evidences for formation of discrete solid phases.

The work of Hallman (12) is of particular interest because of its close relationship to the present study. He claimed preparation of a hydrated magnesium aluminate species from strongly alkaline aluminate solution (pH 12–13) and magnesium sulfate having the following formula

$$[Mg(OH)]_4\ [(HO)_4Al \overset{OH}{\underset{OH}{}} Al(OH)_4]$$

The work of Cole and Hueber (11) indicated that aluminate concrete long exposed to sea water formed $4MgO \cdot Al_2O_3 \cdot xH_2O$ and $4MgO \cdot 2Al_2O_3 \cdot MgSO_4 \cdot xH_2O$.

In general, the past studies suffered from the fact that compositional determinations were based on analysis of washed samples of the precipitated phases. Since many of these systems were subject to hydrolytic changes during washing, these analytical findings did not reflect the actual composition of the precipitates. For the present investigations, use was made of the phase solubility technique developed earlier for systems of this type (18) which permitted determination of the true nature of the precipitated phase.

EXPERIMENTAL

Reagents

Only reagent grade chemicals were used except for the indicators which were utilized as received. The water used in these studies was made by distillation from all borosilicate still of ordinary distilled water. For phase solubility studies this was boiled and cooled under nitrogen prior to use.

Preparation of Sodium Aluminate Solution.—The formula used for 0.2 *M* stock solution was: $Al(OH)_3 \cdot nH_2O$ (Fisher Scientific Co.), 15.22 Gm., sodium hydroxide, reagent grade, 25.00 Gm., and enough distilled water to make 1000 ml.

Procedure.—The sodium hydroxide was heated in a nitrogen atmosphere to a melt in a large nickel crucible. Aluminum hydroxide was added with stirring to the melt in small divided portions with a nickel spatula. After completion of the addition, the mixture was stirred thoroughly and cooled to room temperature under nitrogen. About 100 ml. of distilled water was added to the resulting sodium aluminate and heated to effect complete solution. The solution was allowed to cool and was made to one liter with distilled water and stored in a polyethylene bottle.

Methods of Analysis

Gravimetric Determination of Aluminum (19) and Magnesium.—The method is based on the fact that aluminum forms an insoluble complex (pK_{sp} = 29.0) with 8-hydroxyquinoline quantitatively in the pH range of 5 and 10, whereas the magnesium complex is insoluble and forms only in alkaline pH, preferably 10. Thus, this method can be used to determine aluminum, in the presence of magnesium, and magnesium as well.

Procedure.—The sample containing aluminum and magnesium (10 to 50 mg. of each) was acidified with hydrochloric acid in a 400-ml. beaker. To this solution was added 5 to 10 ml. of 10% tartaric acid solution (10 × wt. of aluminum) to keep the aluminum in solution, followed by 15 ml. of a 30% ammonium acetate solution. After dilution with distilled water to 300 ml., the pH was adjusted with 2 *N* ammonia to 5.2 to 6.8 with the aid of 3 drops of a bromocresol purple indicator solution. The solution sample was warmed, and then 5 ml. of a 50% 8-quinolinol in glacial acetic acid was pipetted into the beaker with stirring. Four or five milliliters of isopropanol was added in some instances to help contain the precipitate in the beaker. The system was heated to nearly boiling and allowed to digest from 0.5–1.0 hour at the temperature of the steam bath, or until the precipitate had become hard and crystalline. During the process of digestion, if the supernatant solution turned pale greenish yellow, more precipitant was added until it turned a definite yellowish orange. At this time the system was cooled to 60° and filtered into a tared 30-ml. glass crucible fitted with a fritted disk of medium porosity. The precipitate was washed with about 75 ml. of distilled water and dried at 135–145° to constant weight (about 2–4 hours). After cooling for one hour in a desiccator, the precipitate was weighed as the anhydrous complex with a molecular weight of 459.42 Gm. per mole.

The determination of magnesium was carried out by collecting the filtrate and the washings from the aluminum determination and placing it into a 600-ml. beaker to which were added 25 ml. of strong ammonia (to give pH 10) and 5–15 ml. of saturated 8-quinolinol in isopropanol. The remaining procedure was the same as given above, except that the precipitate was washed with a 30% isopropanol solution containing 1% ammonia. The precipitate was weighed as the anhydrous complex with a molecular weight of 312.60 Gm. per mole.

EDTA Titration of Magnesium.—The direct titration of magnesium in solution presented no difficulties except that the rate of reaction between EDTA and magnesium-Eriochrome Black T complex (20, 21) and magnesium-Calmagite complex was rather low at room temperature. To overcome this difficulty, the titration was carried out at 60°. Even though Lindstrom and Diehl (22) claimed that Calmagite was more stable than Eriochrome Black T when both were 0.1% aqueous solutions, these indicators were best used as 1% triturations in either sodium chloride or potassium chloride and stored in well stoppered bottles.

Lewis and Melnick (23) studied the accuracy in the determination of calcium and magnesium with EDTA and found that the magnesium titration at pH 10 was complete when a permanent blue color developed. When this end point was used, a satisfactory agreement was obtained for EDTA solutions standardized separately with four different

elements, the results agreeing within about one part per thousand.

Procedure.—A 5.0- to 25.0-ml. sample of the magnesium solution was pipetted into a 125-ml. Erlenmeyer flask and sufficient double distilled water was added to give a 45-ml. total volume. This was followed by 5 ml. of 0.1 *M* borate pH 10 buffer solution and heating on steam bath to 60°. After addition of an estimated 10–15 mg. of the indicator trituration, the sample was titrated slowly until all traces of the red tint had just disappeared and a permanent blue color appeared. The amount of magnesium was calculated: one ml. 0.0100 *M* EDTA = 0.2432 mg. of magnesium.

Precipitation of Sulfate as Barium Sulfate.—The method was that described by Blaedel and Meloche (24), but was tested for accuracy and precision in the experimental concentration range. The result for the average of four determinations of a 5.000×10^{-3} mole per liter of sodium sulfate solution was $4.99 \pm 0.05 \times 10^{-3}$ *M*.

Special Equilibration Procedure Used in the Phase Studies

Short Equilibration Procedure—Beaker Method for Determining Acetic Acid Requirements.—A twenty-milliliter aliquot of the 0.1 *M* sodium aluminate solution was pipetted into a 150-ml. beaker containing a teflon encased magnetic bar and distilled water (sufficient to make up to approximately 100 ml. final volume) in a nitrogen atmosphere. To this was added with constant stirring 8.71 ml. of 1.042 *N* acetic acid containing varying amounts of 0.2 *M* magnesium sulfate. The final pH was measured after the system had apparently approached a steady value (about one-half hour). The precipitate was then quantitatively transferred onto a 60-ml. fritted disk glass filter, and washed with 75 ml. of distilled water. The precipitate was dissolved with sufficient concentrated hydrochloric acid and followed by several portions of distilled water. The filtrate was collected in a 400-ml. beaker. The aluminum and magnesium contents of the filtrate were determined by the 8-quinolinol precipitation method.

Long Equilibration Procedure Bottle Method for Determining Acetic Acid (or Sodium Hydroxide) Requirement.—Preliminary potentiometric titrations served as guides in spacing the amount of 0.1 *M* acids or acidic compounds to be used. The preselected volume of 0.1 *M* titrant was accurately measured into a 120-ml. wide mouth bottle equipped with a polyethylene lined screw cap. Sufficient distilled water was added to give 95.0 ml. before 5.0 ml. of the 0.1 *M* sodium aluminate solution was added from a 10-ml. autoburet to give the 100-ml. final volume. After the cap was secured on tightly, the system was equilibrated by slow rotation in a constant temperature bath set at 30° for two days or longer. At the end of this period the bottles were allowed to stand overnight in the bath to permit the fine precipitates to settle leaving a clear supernatant liquid. The screw cap was removed after careful drying and the pH of the supernate was measured.

Bottle Method—Phase Study of Unbuffered Systems.—The predetermined amounts of distilled water, 0.1 *M* sodium sulfate, 0.1 *M* acetic acid, or 0.1 *N* sodium hydroxide (as necessary to yield the approximate final desired pH value), 0.1 *M* magnesium acetate, and 0.1 *M* sodium aluminate solution were measured accurately in order as listed into a 120-ml. bottle to give a total final volume of 100 ml. A series of such bottles was prepared at one time. These prepared samples were rotated at 30° for a period of five days or longer at 30 r.p.m., after which time the same procedure was followed as given in the preceding section. Mixtures containing definite precipitates were filtered through sintered glass filters, whereas those containing colloidal particles were filtered through membrane filters of appropriate pore sizes. The highest pH value attainable after a little agitation was recorded as indicated by a glass electrode. Aliquot portions of the solutions were taken for chelometric determination of magnesium and gravimetric analysis of sulfate.

Bottle Method—Phase Study of Buffered Systems.—The procedure used was essentially the same as for unbuffered systems except that part of the distilled water was replaced by a buffer solution. Either 0.1 *M* acetic acid or 0.1 *N* sodium hydroxide solution was used to adjust the system approximately to the desired pH before buffering at this pH. The volume of the buffer was such that when diluted to the final volume of 100 ml. the resulting solution gave the desired pH reading. The buffers consisted of either 0.2 *M* of ammonia or 0.2 *M* of tris-(hydroxymethyl)-aminomethane with various amounts of 0.2 *M* hydrochloric acid.

EXPERIMENTAL OBSERVATIONS

Results of experimental studies on hydrous magnesium aluminate systems formed in the presence of sulfate, acetate, and sodium ions are reported. In general, the methods of investigations utilized solu-

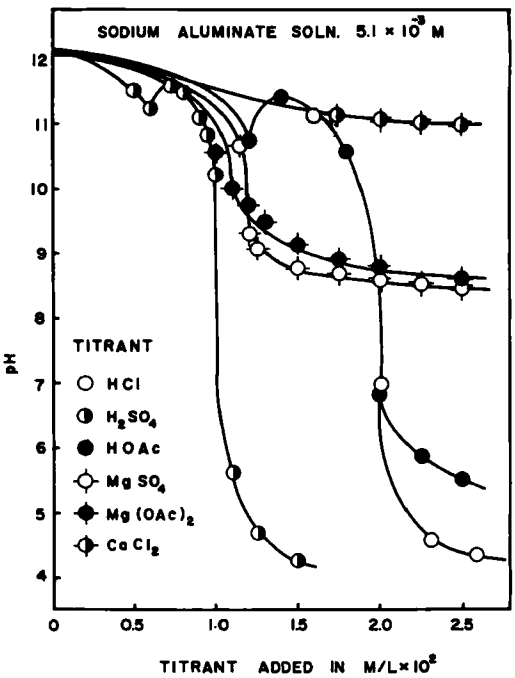

Fig. 1.—Changes in pH of a sodium aluminate solution containing an excess of sodium hydroxide as elicited by several acids and acidic salts.

TABLE I.—PHASE SEPARATION IN THE TITRATION OF SODIUM ALUMINATE SOLUTION BY VARIOUS ACIDS AND ACIDIC SALTS

Titrant	Gross Appearance	Max. pH
HCl	Pptn.	11.2
HOAc	Pptn.	11.4
H_2SO_4	Pptn.	11.6
$MgSO_4$	Pptn.	11.9
$Mg(OAc)_2$	Pptn. colloid	11.9
		11.8
$CaCl_2$	Pptn.	11.9

tions of sodium aluminate, magnesium sulfate, magnesium acetate, and acetic acid to achieve the desired end states. Preliminary investigations necessary in obtaining the proper ratios of reactants to yield systems having comparable end pH values are reported initially.

pH and Acid-Base Relationship

Changes in pH of Sodium Aluminate Solution Elicited by Slow Addition of Acids and Acidic Salts.—Because of its amphoteric nature, aluminum can exist in solution under either highly alkaline or acidic conditions. If a highly alkaline aluminate solution is progressively made acidic by additions of acids or acidic salts such as magnesium sulfate, magnesium chloride, magnesium acetate, or calcium chloride, aluminum is precipitated simultaneously as the pH decreases. Hence, a considerable insight into the mechanism of the precipitation process can be gained from such potentiometric titration plots.

Figure 1 shows the titration characteristics of a sodium aluminate solution (containing an excess of sodium hydroxide with a mole ratio of NaOH:Al $(OH)_3$ of approximately 3.9) when titrated with hydrochloric acid, acetic acid, sulfuric acid, magnesium sulfate, magnesium acetate, and calcium chloride. The bottle technique for potentiometric titration under equilibrium conditions was utilized in obtaining these results. Each bottle contained 0.51 mmole of aluminate plus a definite quantity of titrant (varying in the series of bottles) in a total volume of 100 ml. The systems were equilibrated at 30° for two days, after which the pH of the supernatant liquid was determined by use of an alkaline range glass electrode.

Potentiometric titration at a normal rate of addition of 0.1 *N* sulfuric acid under a nitrogen atmosphere produced erratic pH readings with considerable drift in values. This phenomenon may be explained by the fact that aluminum can exist in aqueous solutions, depending on the medium, in several different forms of aquo-hydroxy species. A change in the medium necessitates a readjustment of the involved equilibria in accordance with the mass law. For this reason the potentiometric readings were obtained under at least pseudoequilibrium conditions.

From the plots depicted in Fig. 1 it is evident that in the highly alkaline pH range both acids and magnesium salts were effective in depressing the pH and in precipitating the aluminate solution. The maximum pH values at which the solid phase just appeared in each system are given in Table I.

Change in pH Produced by Fast Addition of Magnesium Sulfate to Sodium Aluminate Solution.—The effect on the hydrogen ion concentration elicited by different rates of incremental addition of magnesium sulfate to the sodium aluminate solution is clearly evident in Fig. 2. In the beaker method potentiometric readings were taken approximately thirty minutes following the addition. In the bottle method, pH determination was made after two days of equilibration under constant agitation. In all cases the system contained a total of four mmoles of aluminum.

The longer equilibration period gave a much sharper potentiometric break. It also produced markedly greater degrees of precipitation of aluminum at higher pH values, nearly all of the trivalent element being removed from solution by the bottle procedure at pH 11, whereas very little precipitation occurred at this pH by the beaker method. This difference may be due to several factors, one of which is the gradual hydrolysis of the aluminate ion (which hydrolyzes slowly in aqueous alkaline solutions). Another possibility is that the sulfate ions may slowly exchange with the hydroxyl ions in forming a new solid phase, thus raising the pH. By both procedures, however, it would appear that nearly all aluminum is precipitated at pH of 8.5.

Acetic Acid Required to Attain Fixed pH in Formation of Hydrous Magnesium Aluminate in Presence of Sulfate.—In forming hydrous magnesium aluminate in presence of sulfate ions from sodium aluminate, acetic acid was used to neutralize the excess sodium hydroxide and to adjust the system to give an essentially constant final solution

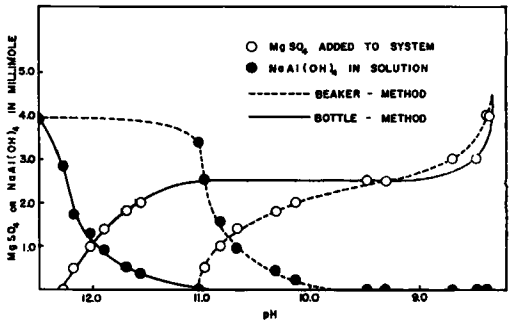

Fig. 2.—Difference in behavior between the beaker and the bottle methods for the precipitation of aluminate by magnesium sulfate as a function of pH.

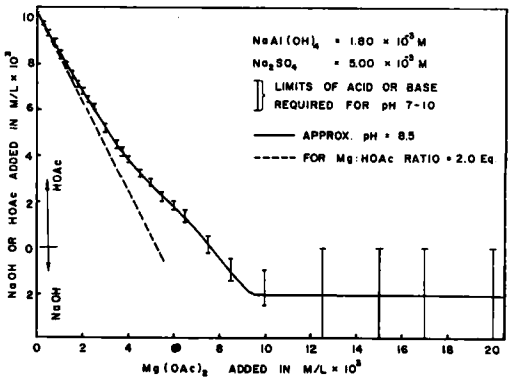

Fig. 3.—Acetic acid or sodium hydroxide–magnesium acetate relationship in systems containing aluminate and sulfate.

pH. It was desirable to maintain the hydrogen ion concentration by addition of acetic acid somewhat above that necessary to prevent precipitation of a discrete magnesium hydroxide phase (*ca.* pH = 10). For this purpose the amount of the acid needed to attain an approximate pH of 8.5 was determined for a system containing a fixed amount of sodium aluminate, sodium sulfate, and varying amounts of magnesium acetate. The bottle method was used, and the system was equilibrated for five days or longer at 30° before the pH of the clear supernatant liquid was determined.

Figure 3 gives the amount of acetic acid or sodium hydroxide added in moles per liter to yield an approximate final pH of 8.5 for various amounts of added magnesium acetate in a system containing 1.80×10^{-3} moles per liter of aluminate. It is evident again that in this pH range the amount of required acid is progressively decreased by addition of magnesium ions. The dotted line corresponds to a stoichiometric ratio of magnesium ion to acetic acid of 2.0. Although magnesium hydroxide is not precipitated as such, it would appear that at low magnesium ion concentration it becomes bound effectively as the oxide to the aluminum system to release two equivalents of hydrogen ions. The same intensity of magnesium ion binding appears to persist up to about two molar equivalents of aluminum present ($2 \times 1.80 \times 10^{-3}\ M = 3.60 \times 10^{-3}\ M$). Beyond this point the binding tendency appears to decrease but still persists noticeably until approximately 12.6×10^{-3} moles per liter of hydrogen ions are released from 1.80×10^{-3} moles per liter of aluminate in the presence of excess magnesium ions $\left(\frac{H^+}{Al} = 7.0\right)$.

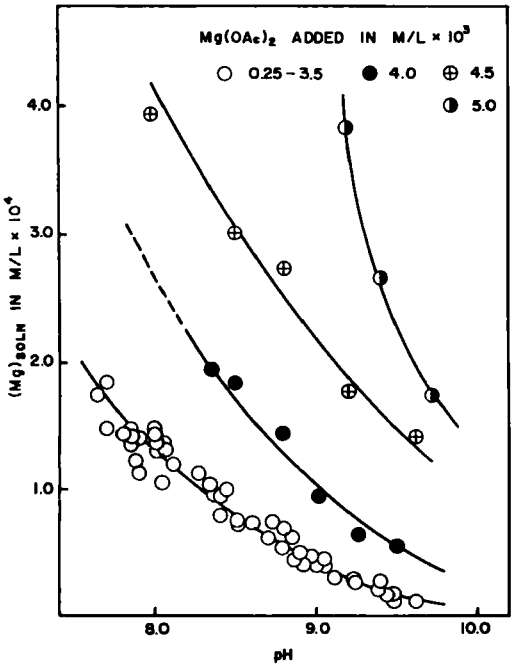

Fig. 4.—Changes in residual magnesium ion concentration in solution elicited by $1.80 \times 10^{-3}\ M$ aluminate as a function of pH for several values of added magnesium acetate in presence of $5.0 \times 10^{-3}\ M$ sodium sulfate.

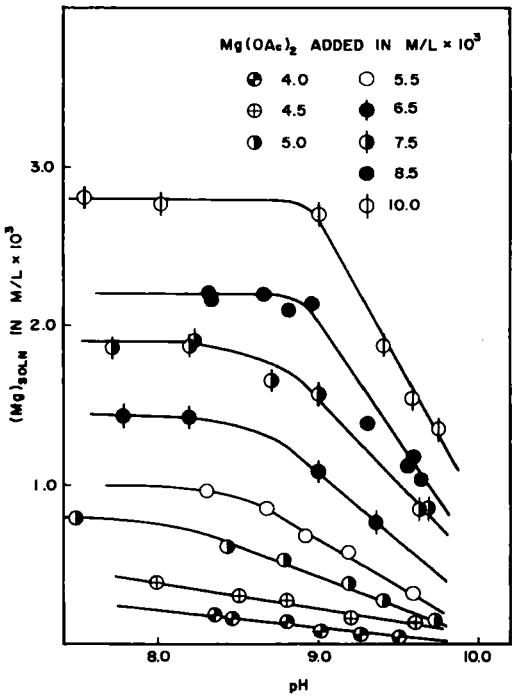

Fig. 5.—Changes in residual magnesium ion concentration in solution elicited by $1.80 \times 10^{-3}\ M$ aluminate as a function of pH for several values of added magnesium acetate in presence of $5.0 \times 10^{-3}\ M$ sodium sulfate.

Phase Solubility Studies on Unbuffered Systems

For the system involving formation of complex crystalline hydrated magnesium aluminate species from solutions of sodium aluminate, magnesium acetate, sodium sulfate, and acetic acid (or sodium hydroxide), considerable information relative to the roles played by each component can be obtained from phase solubility diagrams of various types. In the succeeding sections the influence of concentration of individual components on the extent of reaction of others are reported. All of the studies were carried out under conditions permitting establishment of equilibrium or pseudoequilibrium relationships.

pH and Degree of Magnesium Ion Binding.—Figures 4, 5, and 6 show the extent of magnesium ion binding exhibited by $1.80 \times 10^{-3}\ M$ aluminate solution containing $5.0 \times 10^{-3}\ M$ sodium sulfate. Figure 4 covers the range of added magnesium acetate of $0.25\text{–}5.0 \times 10^{-3}\ M$; Fig. 5 from 4.0–10.0 $\times 10^{-3}\ M$ and Fig. 6 from $12.5\text{–}20.0 \times 10^{-3}\ M$. The pH values were those obtained by the addition of appropriate amounts of acetic acid or sodium hydroxide. Any reduction in solution concentration below the slope of one is, of course, due to binding in these plots.

The data interpolated from the plots were used to construct Figs. 7 and 8. These phase solubility diagrams can be interpreted in terms of the classical phase rule. According to Fig. 7, the presence of less than the atomic ratio of Mg:Al of 2.0 led to essentially an invariant concentration of magnesium ion in the supernatant liquid, suggesting the presence of two discrete solid phases over this plateau

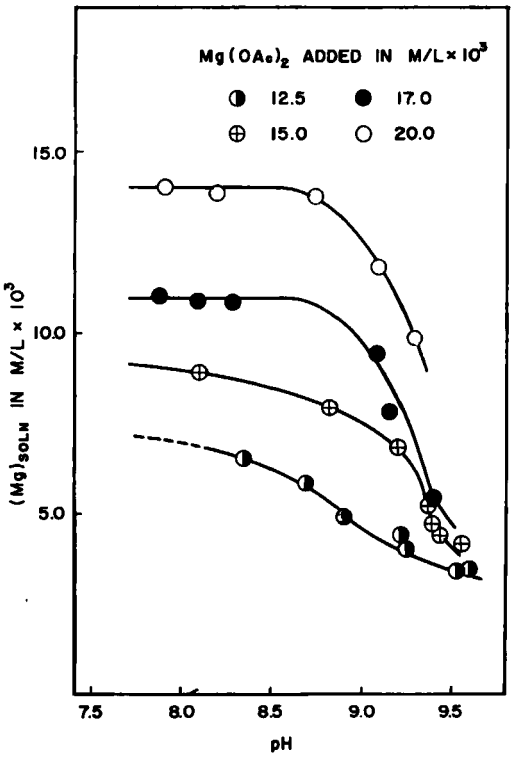

Fig. 6.—Changes in residual magnesium ion concentration in solution elicited by 1.80×10^{-3} M aluminate as a function of pH for several values of added magnesium acetate in presence of 5.0×10^{-3} M sodium sulfate.

range. An increase in the added magnesium concentration above this point (3.60×10^{-3} M) resulted in a sharp rise in the magnesium ion concentration in solution. The slope at this point was, however, well below one. Further increase in the amount of added magnesium ion eventually caused a corresponding rise in solution concentration as shown in Fig. 8. Each dotted line extrapolated to a solid phase with an atomic composition of four magnesium to one of aluminum.

The influence of pH on the phase solubility diagrams was noticeable but surprisingly small. This was particularly true at pH values below 9 and with higher added magnesium acetate concentrations.

The results presented were obtained by use of the bottle method as described in the section on experimental procedure for phase solubility studies in unbuffered systems. Predetermined amounts of the components were added to each bottle in a definite order. The aluminate solution used contained a large excess of sodium hydroxide with a sodium hydroxide to aluminum hydroxide molar ratio of approximately 5.7. The systems were equilibrated at 30° for five days or longer after which the pH and the magnesium concentration in the supernatant liquid were determined.

In summary, the phase solubility diagrams obtained have indicated formations of at least two crystalline forms of $m\mathrm{MgO}\cdot n\mathrm{Al_2O_3}\cdot p\mathrm{SO_3}\cdot x\mathrm{H_2O}$. The first had an atomic ratio of Mg:Al of 2.0 and the second had a ratio of 4.0. The very flat plateau corresponding to the formation of the first suggested that the structure of this species was such as to permit ready formation of well-defined crystals. On the other hand, the second corresponded possibly to a limiting structure where definite crystals formed but might have permitted partial replacement by magnesium ions. Only in the limiting case would the ratio of 4.0 be achieved.

Effect of Sulfate Concentration on the Degree of Magnesium Binding.—A review of Table I indicates that sulfate was necessary in the formation of solid phases in reactions of magnesium ion with aluminate. The reaction between magnesium acetate and sodium aluminate invariably produced a colloid which could be coagulated, besides sulfate, by many other polyvalent anions. For this reason sulfate was used in excess but constant amount (5.0×10^{-3} M) in the previous phase solubility studies.

Some experimental data to show the effect of added sulfate concentration on the degree of magnesium binding exhibited by 1.80×10^{-3} M aluminate for three initial magnesium acetate values have been compiled in Table II. Figure 9 gives the plot of magnesium solution concentration *vs.* the amount of added sodium sulfate and the colloid–precipitation range as well. Figure 10 shows the reciprocal relationship of added sodium sulfate concentration on the degree of magnesium binding for three values of added magnesium acetate. Although the pH values of these systems ranged rather widely, the averages were 8.6, 8.7, and 8.6 for the

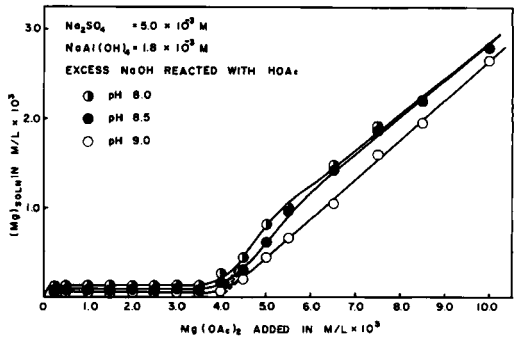

Fig. 7.—A phase solubility diagram showing the degree of magnesium ion binding exhibited by aluminate in presence of sulfate for three pH values.

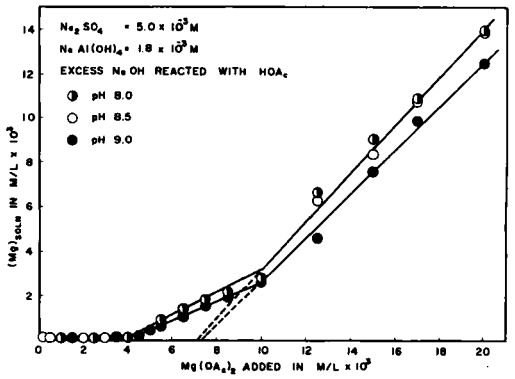

Fig. 8.—A phase solubility diagram showing the degree of magnesium ion binding exhibited by aluminate in presence of sulfate for three pH values.

TABLE II.—EFFECT OF SULFATE CONCENTRATION ON THE RESIDUAL CONCENTRATION OF MAGNESIUM ION IN SOLUTION FOR THREE ADDED MAGNESIUM ACETATE VALUES

Na_2SO_4 added in $M/L \times 10^4$	Residual Magnesium in Solution in $M/L \times 10^4$: 36.0×10^{-4} M added	75.0×10^{-4} M added	150.0×10^{-4} M added
0.00	4.939	19.063	87.214
2.50	3.161	19.063	82.275
5.00	1.185	17.890	81.288
7.50	0.504	17.387	80.794
10.00	0.000[a]	17.087	80.596
15.00	0.346	16.445	80.498
25.00	0.049	17.384	80.201
50.00		16.988	79.806

[a] pH on this run was allowed to rise considerably above those of the others. Added aluminate = 18.0×10^{-4} M, pH adjusted to the average values of 8.6, 8.7, and 8.6 with either acetic or sodium hydroxide for the systems of added magnesium acetate 36.0, 75.0, and 150.0×10^{-4} M, respectively.

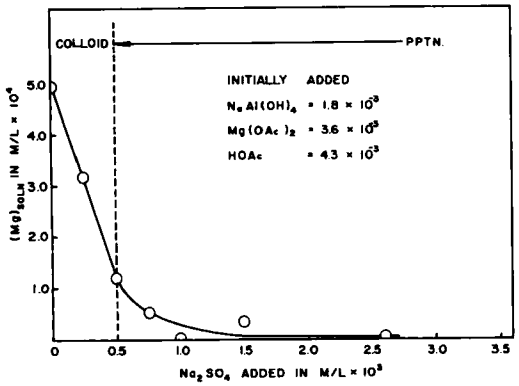

Fig. 9.—Changes in residual magnesium ion concentration exhibited by aluminate as a function of added sodium sulfate concentration in systems with an average pH value of 8.6.

added magnesium acetate of 3.60, 7.50, and 15.0×10^{-3} M, respectively.

With respect to the amount of magnesium bound in the precipitate, Fig. 10 shows that in the presence of excess sulfate ions the maximum atomic ratio of Mg:Al in the solid phase varied with added magnesium acetate as follows: magnesium acetate added in $M/L \times 10^3$, 3.60, 7.50, and 15.0, maximum Mg:Al atomic ratio in solid phase, 2.0, 3.3, and 3.9.

pH and Degree of Sulfate Binding.—The experimental values showing the effect of pH on the degree of sulfate binding as exhibited by 1.80 and 3.60×10^{-3} M of aluminate for various amounts of added magnesium acetate have been plotted in Figs. 11 and 12. The former covers the pH range of 7.5–10.0 and the latter from 9.0–14. The pH values were those elicited by the addition of appropriate amounts of acetic acid or sodium hydroxide.

Figure 11 indicates that pH had no apparent effect on the degree of sulfate binding in this range. The degree of sulfate binding seemed to approach a limiting value (*ca.* $0.8–0.9 \times 10^{-3}$ M) when the added magnesium acetate concentration was equal to or greater than 4.0×10^{-3} M in a system that contained 1.80×10^{-3} M of aluminate. This corresponds roughly to Al:sulfate ratio of 2.0. Figure 12 shows essentially the same effect for pH values below 11.0 with the exception that the degree of sulfate binding had not reached a limiting value. At pH values greater than 11.0 the degree of sulfate binding decreased until no binding was exhibited at pH 12.5 or greater for a system containing 3.60×10^{-3} M of aluminate.

Effect of Sulfate Concentration on the Degree of Sulfate Binding.—The effect of increasing sulfate concentration on the amount of sulfate bound by the hydrous magnesium aluminate system is evident from Fig. 13. The apparent extent of sulfate binding appears to be essentially independent of the sulfate concentration when even a slight excess of the divalent ion was present. The amount of binding appears to correspond to slightly less than that expected on the basis of Al:sulfate ratio of two, particularly at low initial aluminate concentration.

The open circles in Fig. 13 represent systems containing 1.80×10^{-3} M aluminate and added magnesium acetate equal to or greater than 5.0×10^{-3} M. The half circles represent systems containing 3.60×10^{-3} M aluminate and added magnesium acetate equal to or greater than 8.0×10^{-3} M. The solid circles represent systems containing 5.10×10^{-3} M aluminate and added magnesium acetate (or magnesium sulfate) equal to or greater than 12.0×10^{-3} M. The technique described in the experimental procedure for phase solubility studies on buffered systems was utilized in this series of experiments. The exceptions were that the sulfate (added either as sodium or magnesium salt) concentration was the variable and that

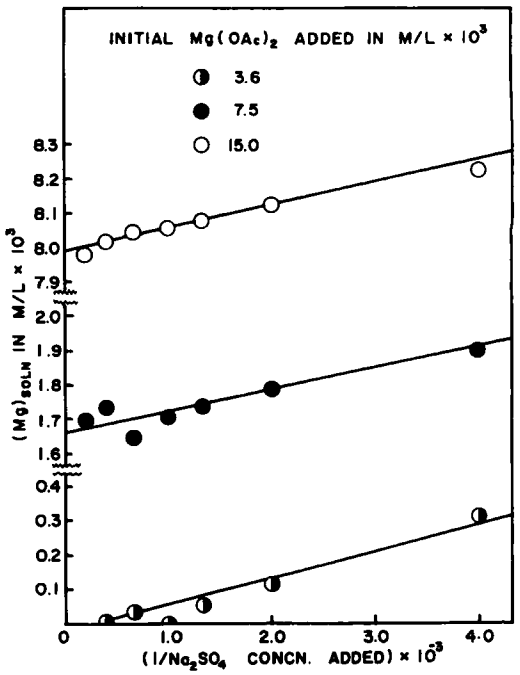

Fig. 10.—Changes in residual magnesium ion concentration exhibited by 1.80×10^{-3} M aluminate as a reciprocal function of added sulfate concentration for three values of added magnesium acetate in systems with average pH values of 8.6–8.7.

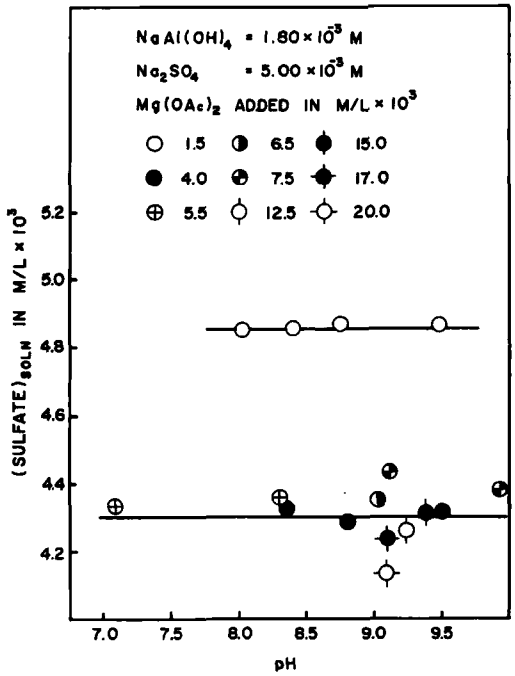

Fig. 11.—The effect of pH on the residual sulfate concentration in solution in systems containing aluminate and different values of magnesium acetate.

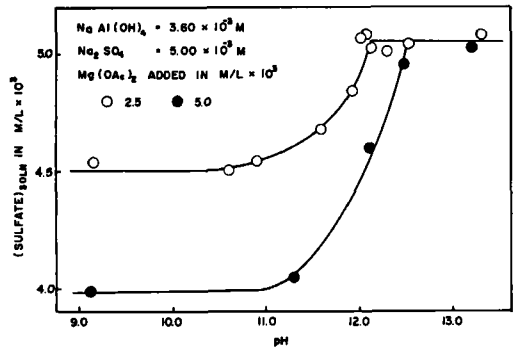

Fig. 12.—The effect of pH on the residual sulfate concentration in solution in systems containing aluminate and different values of magnesium acetate.

the system contained an excess amount of magnesium ions. At the end of the specified duration and conditions of equilibration, a 50-ml. aliquot sample of the filtered supernatant liquid was analyzed for the residual sulfate concentration according to the gravimetric method given earlier.

A study of Fig. 13 shows that the slopes of the three straight lines are all essentially in unity, indicating that further addition of sodium sulfate to the system caused only a corresponding increase in solution concentration. Under these experimental conditions the intercepts obtained by extrapolation of the linear portion of each curve provided the necessary composition information for the case of sulfate binding in the presence of excess of magnesium ions in solution. The results, when expressed as the atomic Al:sulfate ratios, are 2.5, 2.1, and 1.9, respectively, for the given aluminate concentrations.

Effect of Magnesium Concentration on the Degree of Sulfate Binding.—Figure 14 shows the extent of binding of sulfate ions as a function of the added magnesium acetate concentration by systems containing 1.80, 2.55, 3.60, 5.10 × 10^{-3} *M* of aluminate, and 0–20.0 × 10^{-3} *M* of added magnesium acetate. These results are shown plotted for three initial concentrations of added sodium sulfate (2.5, 5.0, and 10.0 × 10^{-3} *M*).

Even a cursory examination of Fig. 14 reveals that all the curves appear to have several manifestations in common, chiefly in the form of linear dependency portions sandwiched between initial and final invariant plateaus relating the effects of added magnesium acetate concentration on sulfate binding.

The addition of small amounts of magnesium ions apparently did not produce sulfate binding. This region probably corresponds to the initial rise in magnesium concentration in solution as shown in Fig. 7. As more magnesium acetate was added, the sulfate ions reacted in the stoichiometric ratio of 4 Mg:1 sulfate, the linear dependency finally leveling off into an invariant plateau. This strongly suggests

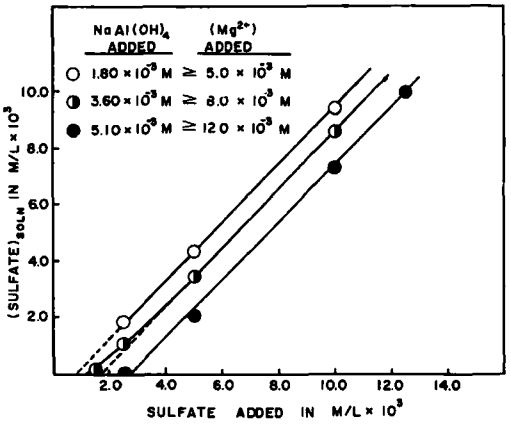

Fig. 13.—A phase solubility diagram showing the extent of sulfate binding exhibited by three concentrations of aluminate in the presence of excess of magnesium ions.

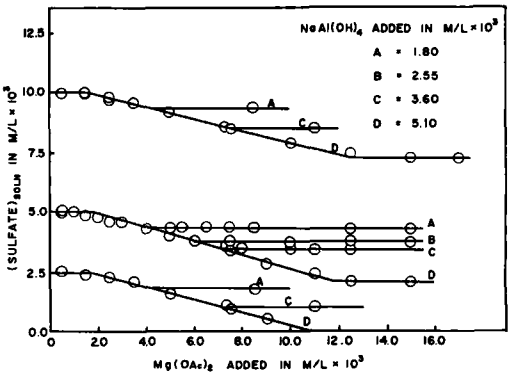

Fig. 14.—Changes in residual sulfate concentration in solution exhibited by several values of aluminate as a function of added magnesium acetate concentration for three added sodium sulfate concentrations.

that after the breakpoint sulfate was not being bound upon further addition of magnesium acetate. Figure 14 shows that these breakpoints depended mainly on the initially added sodium aluminate concentration. The stoichiometry at this point was approximately $4MgO \cdot Al_2O_3 \cdot SO_3 \cdot yH_2O$.

It should be kept in mind, however, that as indicated in Fig. 8, additional magnesium was taken up beyond the breakpoint. Eventually the final stoichiometry, as obtained in the presence of the large excess of magnesium ions indicated in a previous section, corresponds to $8MgO \cdot Al_2O_3 \cdot SO_3 \cdot zH_2O$. Since the Al:sulfate ratio is the same for both forms, Fig. 14 does not show further change in sulfate uptake beyond the breakpoint.

Phase Solubility Studies on Buffered Systems

The results reported in the preceding sections were obtained on systems initially containing only aluminate, acetate, sodium, magnesium, and sulfate ions, other than those contributed by water. Studies were also carried out with the addition of ammonium and chloride ions and ammonia particularly, since their presence permitted closer control of the relative basicity of the aqueous solutions. The behavior of the resulting and similar systems are substantially different from those of the unbuffered mixture and are reported in detail in this section.

Magnesium Binding and Effect of Buffer.—Figures 15 and 16 show the extent of magnesium ion binding exhibited by 1.80×10^{-3} *M* of aluminate containing 5.0×10^{-3} *M* sodium sulfate in presence of a relatively low buffer concentration of 1.0×10^{-2} *M* ammonia buffer (adjusted with hydrochloric acid). Figure 15 covers the range of added magnesium acetate of $0.5\text{–}3.0 \times 10^{-3}$ *M*, and Fig. 16 from $3.5\text{–}8.5 \times 10^{-3}$ *M*. The pH values given were obtained by use of predetermined amounts of acetic acid or sodium hydroxide and the appropriate buffer. The data interpolated from the two plots were then used to construct Fig. 17. The four curves designated in Fig. 17 by open, half solid, and quarter circles represent systems with pH values of 8.50, 8.75, 9.00, and 9.25, respectively. These results were obtained by use of the bottle method as described in the experimental section. Predetermined volumes of solutions of the components were measured into each bottle in a definite order. The addition of buffer simply replaced part of the distilled water so that the final volume remained 100 ml. The aluminate solution used had a sodium hydroxide to aluminum hydroxide mole ratio of approximately 5.7. Other than the specified cases, these systems were equilibrated at 30° for five days or longer, after which the pH and the magnesium concentration in the supernatant liquid were determined.

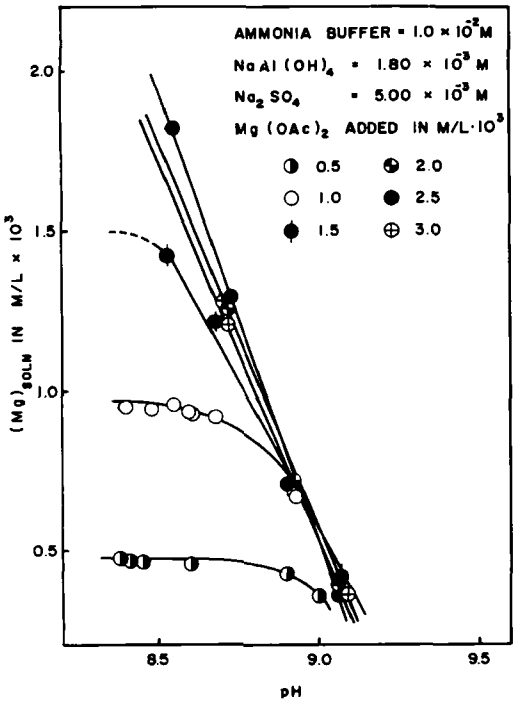

Fig. 15.—Changes in residual magnesium ion concentration in solution elicited by aluminate as a function of pH for several values of added magnesium acetate in buffered systems containing sulfate ions.

The binding behaviors exhibited in the presence of 0.01 *M* ammonia buffer as shown in Fig. 17 differ largely from the unbuffered systems in the significantly higher magnesium concentrations required to form the insoluble species. The analysis of the straight line portions of the plots at high magnesium concentrations yield the relationships

System with pH	Slope of final portion of curve	Extrapolated Mg:Al atomic ratio
8.50	0.98	1.66
8.75	0.93	1.72
9.00	0.88	1.81
9.25	0.77	1.92

The influence of pH is obvious in that a change from 9.25 to 8.50 caused the slopes to approach unity and the extrapolated Mg:Al ratio to assume a lower value. The data shown above suggest that at higher pH values the system behaved much like the unbuffered, whereas at lower pH there is suggestive evidence of formation of another species corresponding possibly to an atomic ratio of three magnesium to two aluminum (or Mg:Al of 1.50). Similar results were also obtained with substantially higher (5×) buffer concentration, as shown in Fig. 18.

The data shown in Fig. 19 also suggest this type of relationship. The determinations in this instance were made with magnesium sulfate instead of the acetate, and variable amounts of the buffer were used. At lower buffer concentrations the results again resemble those from the unbuffered runs. At a higher buffer concentration, particularly with a shorter time of equilibration, the precipitated species correspond to three magnesium to two aluminum (7.6×10^{-3} *M* magnesium to 5.1×10^{-3} *M* aluminum). This suggests that the 3:2 species may be metastable relative to the 2:1 form.

Similar response to pH was exhibited when another buffer species was employed. Figure 20 shows the relation obtained when tris-(hydroxymethyl)-aminomethane (THMAM) was used as the buffering agent with two days of equilibration period. In Fig. 21 the corresponding plots of precipitated magnesium *vs.* the amount added in the form of magnesium sulfate is shown. At lower pH values it appears

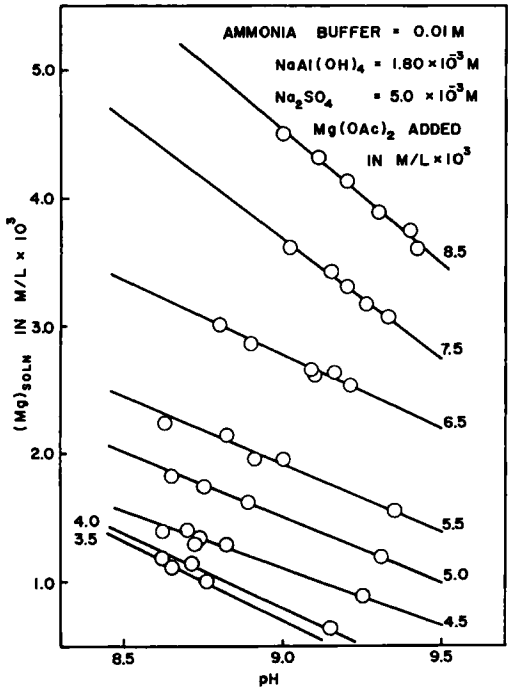

Fig. 16.—Changes in residual magnesium ion concentration in solution elicited by aluminate as a function of pH for several values of added magnesium acetate in buffered systems containing sulfate ions.

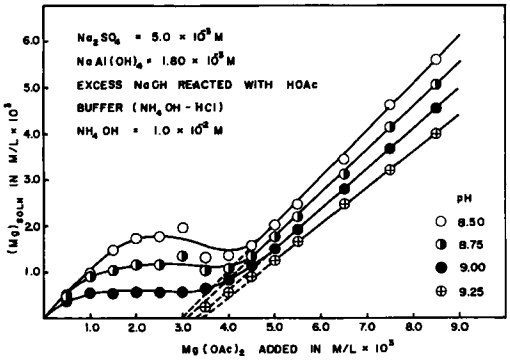

Fig. 17.—A phase solubility diagram showing the extent of magnesium ion binding exhibited by aluminate in buffered systems containing sulfate ions for four pH values.

that the break in the bound magnesium occurs at around 7.5×10^{-3} *M* corresponding again to 3:2 ratio. There appears to be another break at 1:2 ratio (2.5:5.1) which was not indicated in the ammonia systems. At higher pH there seems to be again the 2:1 relationship in presence of excess magnesium.

Sulfate Participation in the Buffered Systems.—It is evident from Figs. 19 and 20 that increasing the sulfate concentration reduces the amount of unbound magnesium ions in these systems. Thus, the species formed must again require sulfate ion in their formations. This is particularly evident in the data presented in Fig. 22, where the residual concentration of magnesium has been plotted against the amount of sulfate added.

The influence of the buffer on the sulfate participation is also shown in Fig. 23, where the residual sulfate has been plotted as a function of added magnesium ions. The amount of bound sulfate (the difference between the added and the sulfate in solution) appears to be somewhat less (approximately 16 per cent) than that in the unbuffered system.

Since the formation of the solid species required sulfate, the effect of sulfate concentration in solution on the residual magnesium concentration in solution may be expected to follow the solubility product principle.

As shown in Fig. 24, a logarithmic plot of data given in Fig. 22 indicates that the sulfate concentration varied inversely as the third power of the magnesium ions. In the limit in presence of excess sulfate ions this also suggests that the solid species corresponds to the composition, 3 Mg:2 Al:1 sulfate for this system.

Properties of the Solid Species

X-Ray Diffraction Patterns.—Figure 25 shows the Debye-Scherrer patterns (25) of relative intensities of some of the stronger lines along with their angles of diffraction. The relative intensity was defined

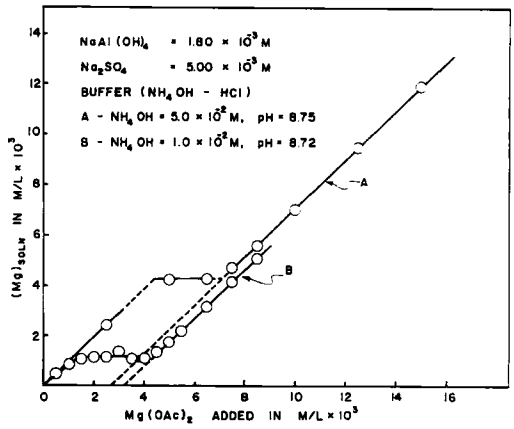

Fig. 18.—A phase solubility diagram showing the extent of magnesium binding exhibited by aluminate in presence of sulfate at two concentrations of ammonia buffer.

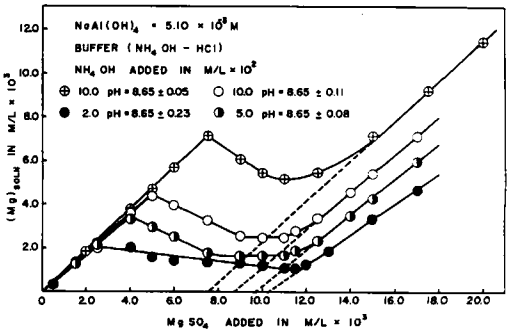

Fig. 19.—A phase solubility diagram showing the extent of magnesium binding exhibited by aluminate at various values of sulfate and three buffer concentrations, equilibration at five days (⊕ = two days).

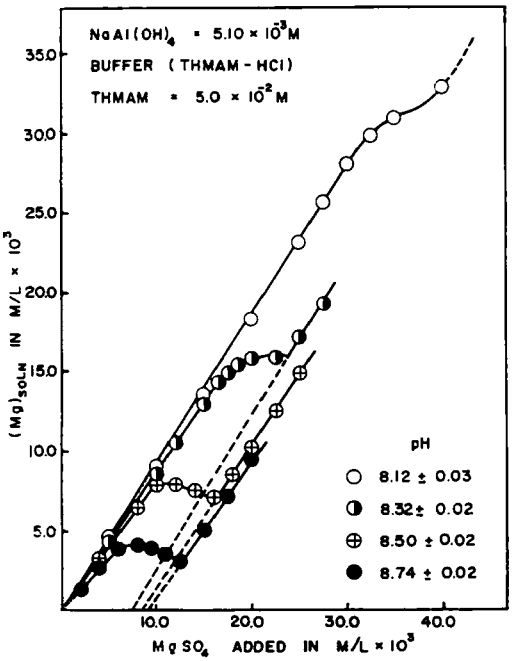

Fig. 20.—A phase solubility diagram showing the extent of magnesium binding exhibited by aluminate in buffered systems at various values of sulfate and pH with two days of equilibration.

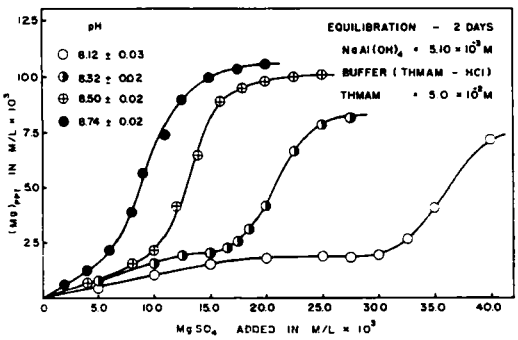

Fig. 21.—The amount of magnesium incorporated in the solid phase as a function of added magnesium sulfate concentration at four pH values in buffered systems containing aluminate with two days of equilibration.

as I/I_1, where I was the peak intensity of a diffraction line as estimated from per cent transmittance of the photographic negative corrected for background, and I_1 was the intensity of the strongest line of the particular phase in question. The diffraction patterns were developed by the use of filtered CuKα(λ = 1.5418 Å.) radiation on dried powdered samples (ground to about 200 mesh) with one hour exposure time on a camera film of 57.3 mm. in diameter. The relative information on the source and state of the powder samples is compiled in Table III. Samples A to E were isolated from phase solubility runs, washed with distilled water, and dried at room temperature and atmospheric pressure. Sample F was made batchwise, washed with distilled water, and dried over concentrated sulfuric acid at a reduced pressure for two weeks.

The effect of composition on the diffraction pattern as shown in Fig. 25 is of some interest. In particular the lines diffracted in the neighborhood of 30 and 33 degrees seemed to be shifted closer toward 30 degrees as the Mg:Al ratios increased. Another significant feature in these several patterns is the presence of an intense doublet at or close to the ten degree angle in samples A, B, and C, but which is either diffused or absent in the remaining samples.

All the samples indicated by the Debye-Scherrer patterns appeared at least partially crystalline and gave similar but significantly different X-ray diffraction patterns. For the series with Mg:Al ratios of 0.5 (A), 1.5 (B), 1.8 (C), and 4.0 (D), the line intensities as a whole increased as the ratios increased with the greatest distortion of lines in sample D (*cf.* Fig. 25).

Although the solid samples subjected to X-ray analysis were recovered directly from phase solubility runs, they were unfortunately subjected to varying degrees of washing with distilled water to remove the residual solution phase, as mentioned. This procedure probably produced significant alterations both in the crystal structures and the chemical compositions of the recovered dried products. Any serious attempts to derive structural information from the diffraction patterns should take this factor into account. The data presented in this section should, therefore, be considered as being indicative rather than conclusive, relative to the true structure of the precipitated species.

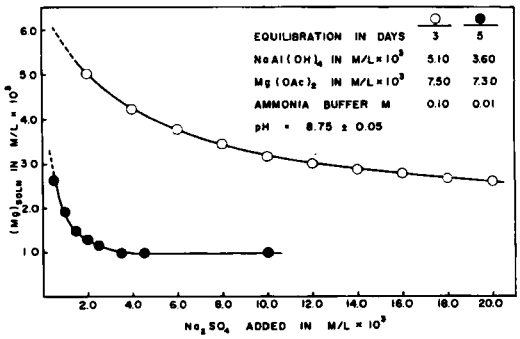

Fig. 22.—Changes in residual magnesium concentration in solution elicited by aluminate as a function of added sulfate concentration in buffered systems designated by open and solid circles.

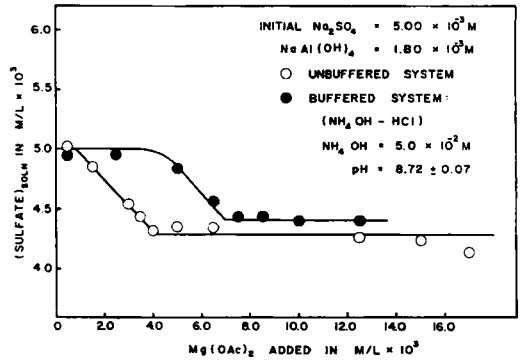

Fig. 23.—Changes in residual sulfate concentration in solution elicited by aluminate as a function of added magnesium acetate concentration in buffered and unbuffered systems.

CONCLUSION

The several solid species presumably formed in aqueous solutions by interactions of sodium, magnesium, aluminate, sulfate, and acetate ions in absence or presence of buffers are listed in Table IV. Their existence is proposed on the basis of experimental results presented in the preceding sections.

The evidence for the existence of species III and IV appears to be quite strong. The formation of species III which appears to be similar to the one reported by Hallmann (12) (who, however, indicated that sulfate was not part of the structure, but rather an impurity in the form of magnesium sulfate) is based on: (*a*) the phase solubility diagram obtained with the unbuffered systems (Fig. 7), (*b*) the potentiometric results given by magnesium acetate (Fig. 3), (*c*) the results from the sulfate study (Fig. 14), and (*d*) X-ray diffraction patterns (Fig. 25).

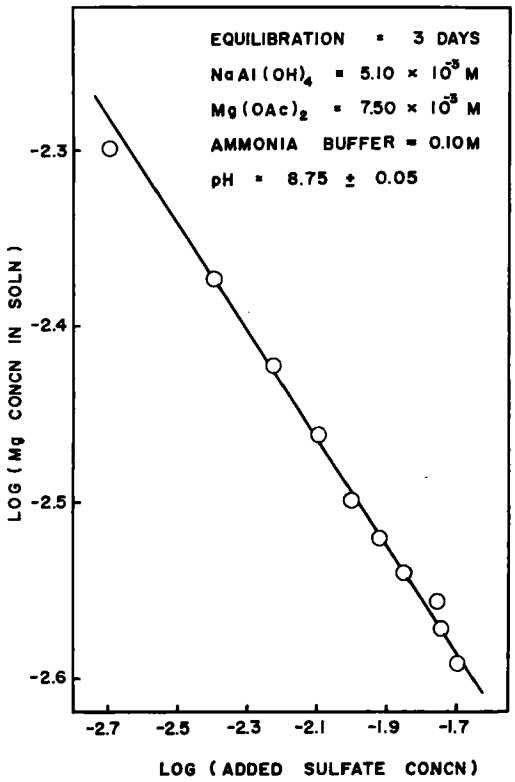

Fig. 24.—Logarithmic relation of the concentrations of residual magnesium ion in solution and added sodium sulfate in buffered systems containing aluminate with three days of equilibration.

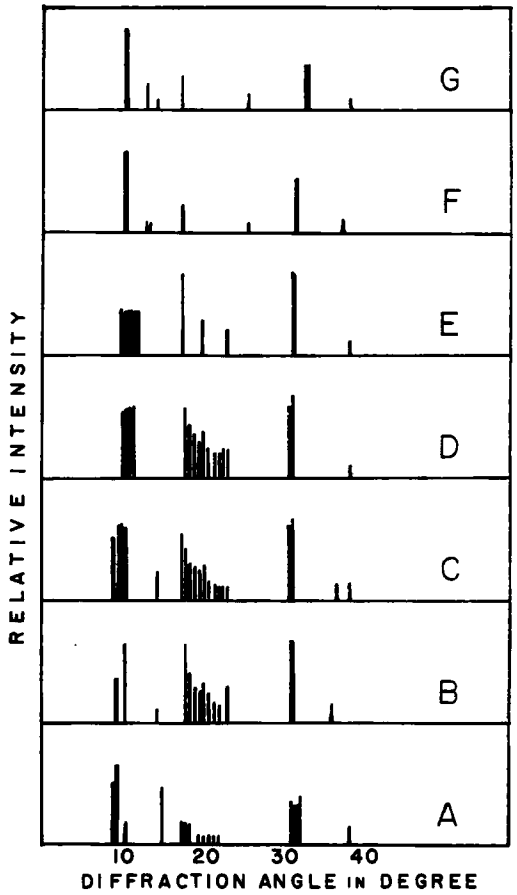

Fig. 25.—Relative intensity and diffraction angle relation of some stronger lines from Debye-Scherrer patterns of several powder samples with various Mg:Al ratios (for source and state see Table III) developed with filtered *CuKα* radiation, one hour exposure, 57.3-mm. diameter camera.

The phase solubility diagram shown in Fig. 7 gives extremely strong support to the existence of solid species III which apparently form in accordance with the phase rule (a solubility product-controlled process as evidenced by an invariant plateau). This certainly obviates the possibility of some of the earlier suggestions that solid solutions are possible over this range.

TABLE III.—INFORMATION RELATING TO THE SOURCE AND STATE OF THE X-RAY DIFFRACTION POWDER SAMPLES OBTAINED FROM PHASE SOLUBILITY STUDIES IN PRESENCE OF SULFATE

Sample	pH	$Mg(OAc)_2$ added in $M/L \times 10^3$	$NaAl(OH)_4$ added in $M/L \times 10^3$	Mg:Al ratio in ppt.
A	8.51	2.5	5.1	0.5
B	8.90	7.5	5.1	1.5
C	7.82	10.0	5.1	1.8
D	8.70	15.0	1.8	4.0
E[a]	8.72	12.5	1.8	1.7
F[b]	8.50	12.5	5.1	1.5

[a] Ammonia buffer = $1.0 \times 10^{-2} M$. [b] Ammonia buffer = $10.0 \times 10^{-2} M$.

TABLE IV.—SOLID SPECIES FORMED IN AQUEOUS SOLUTION BY INTERACTIONS OF SODIUM, MAGNESIUM, ALUMINATE, SULFATE, AND ACETATE IONS IN THE ABSENCE OR PRESENCE OF BUFFERS

Species	Composition	Conditions	Degree of Assurance
I	$MgO \cdot Al_2O_3 \cdot SO_3(?) \cdot nH_2O$	Low pH and high buffer concentration	Uncertain
II	$3MgO \cdot Al_2O_3 \cdot SO_3 \cdot xH_2O$	Low pH and moderate buffer concn.	Fair
III	$4MgO \cdot Al_2O_3 \cdot SO_3 \cdot yH_2O$	Unbuffered or at low buffer concn.	Definite
IV	$8MgO \cdot Al_2O_3 \cdot SO_3 \cdot zH_2O$	Unbuffered in an excess of Mg ion in solution	Definite

The 2 Mg:Al ratio of solid species III was obtained by the extrapolation of the rising portion of the curve to zero residual magnesium concentration in solution.

The composition of species III is also supported by the number of equivalents of hydrogen ion released when magnesium ion became bound through oxide to the aluminum system. Figure 3 shows that the amount of acetic acid required to obtain a pH value of approximately 8.5 in a system containing 1.80×10^{-3} *M* aluminate and excess sulfate is progressively decreased by addition of magnesium ions. The initial intensity of magnesium ionic binding appears to persist up to approximately two molar equivalents of aluminum present. The resulting composition corresponds to an atomic Mg:Al ratio of two to one, or that of solid species III.

Further evidence in support of the existence of species III is provided by the study of the effect of magnesium concentration on the extent of sulfate binding. The breaks in the plots shown in Fig. 14 indicate formation of a solid phase with an approximate 4 Mg:2 Al:1 SO_4 ratio which corresponds to that of species III.

Similar evidence is available in support of the existence of species IV. The phase solubility diagram as given in Fig. 8 provides the strongest indication for the formation of a solid species with the composition corresponding to that of species IV. This solid phase appears to form, however, without the characteristic invariant plateau. This behavior may be rationalized on the basis of a mass-controlled process rather than that of a solubility product. This is not an exception to the phase rule, but a modification with the addition of the mass law to account for the presumed dissociation phenomenon.

Figure 3 gives further support to the existence of solid species IV. The 4 Mg:1 Al ratio is obtained from the number of equivalents of hydrogen ion released when magnesium ions became bound through oxide to the aluminum system and by taking into account the effect of sulfate. The magnesium binding persists until approximately 12.6×10^{-3} mole per liter of hydrogen ions are released from 1.80×10^{-3} *M* aluminate. Since the sulfate effect is equivalent to 1.80×10^{-3} moles per liter of hydrogen ions, the atomic Mg:Al ratio is approximately equal to $(12.6 + 1.8) \div (1.8 \times 2) \doteq 4:1$.

Supplementary evidence in the support of the existence of solid species IV is afforded by the X-ray patterns shown in Fig. 25 for the sample D with a 4 Mg:1 Al ratio which corresponds to that of the composition of species IV. The diffraction lines show the solid sample to be crystalline.

It is to be noted that species IV may be similar to one of the double hydroxides of magnesium and aluminum reported by Feitknecht, *et. al* (13–15). These double hydroxides differ from the present reported species by having a characteristic varying formula in which the sum of the number of atoms of magnesium and aluminum is equal to five, and which may also contain an atom of chlorine.

The evidence in support of the existence of species II is based on: (*a*) phase solubility diagrams obtained with buffered systems (Figs. 17, 18, and 19), (*b*) the results of magnesium and sulfate relation study in the reference system (Fig. 24) and (*c*) X-ray diffraction patterns (Fig. 25). The formation of species II is suggested by the phase solubility diagrams with buffered systems shown in Figs. 17, 18, and 19. The formation of the 3 Mg:2 Al solid species apparently require a combination of low pH value and moderate buffer concentration. The behavior of the system suggests, however, that solid species II may be metastable relative to species III, since a longer period of equilibration, higher pH values, or lower buffer concentration led to the formation of the latter solid phase.

The solubility product relationship as shown in Fig. 24 also indicates the presence of a 3 Mg:1 SO_4 species in this system. Since the system shows 3 Mg:2 Al ratio, the formation of a solid species may be rationalized to have the overall 3Mg:2 Al:1 SO_4 ratio, which corresponds to that of species II.

The Debye-Scherrer pattern of sample F with the Mg:Al ratio of 1.5 corresponding to the composition of the species II indicates that the species in question is at least partially crystalline. The unbuffered sample B and the buffered sample F are similar in composition; the difference in their

X-ray patterns may be due to drying conditions and to the effect of buffer in sample F.

Here again, species II may be similar to one of the double hydroxides reported by Feitknecht, since the number of atoms of magnesium and aluminum in species II does add up to five. However, species II has a sulfate moiety in the formula which the double hydroxides apparently lack.

The evidence citable in support of the existence of species I is based on (*a*) the phase solubility diagram shown in Fig. 20 and (*b*) the X-ray diffraction pattern in Fig. 25. The results of the data shown in the reference diagram indicate that species I required a combination of high hydrogen ion, high buffer, and low residual magnesium ion concentrations. These prerequisites are met in the system as designated by half and open circles in Figs. 20 and 21. Figure 21 shows clearly the presence of an invariant plateau in each of the systems with pH values of 8.12 and 8.32. These two curves indicate approximate atomic Mg:Al ratios of 1:2, which correspond to that of the solid species I.

It should be explicitly pointed out that in these studies no serious attempt was made to determine the extent of participation of singly charged ions other than H^+ and OH^-. Acetate, chloride, sodium, and other ions present probably were coprecipitated to varying degrees. It appears likely, however, that their presence does not seriously alter the qualitative nature of these findings.

The results of the present investigation, nevertheless, appear to show that the hydrous-sulfated magnesium aluminate system could very well exist in a number of solid species with different stoichiometric ratios. They also serve to illustrate the value of phase solubility studies in delineating complex inorganic systems.

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Triterpene Constituents of *Sarcostemma viminale* R.Br.

By JOHN D. TORRANCE and JOHANNES L. C. MARÁIS

The isolation of three triterpenes from a benzene extract of *Sarcostemma viminale* by alumina chromatography is described. Two of the triterpenes were identified as β-amyrin and friedelin, but the third, which occurred in the plant as an acetate, could not be identified with any known triterpene acetate. The monohydroxy-triterpene obtained by hydrolysis of the acetate has been designated viminalol.

SARCOSTEMMA VIMINALE is a leafless, glabrous, fleshy climbing plant belonging to the natural order *Asclepiadaceae*. The plant, which has numerous round-jointed stems and a slightly bitter milky latex, is found throughout Central and Southern Africa (1, 2) where it has been used medicinally by various tribes, both as an emetic and as a galactogogue (3). Steyn (4) found *Sarcostemma viminale* to be poisonous, and the plant is thought to be responsible for stock losses in southwest Africa (5).

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Triterpene Constituents of *Sarcostemma viminale* R.Br.

By JOHN D. TORRANCE and JOHANNES L. C. MARÁIS

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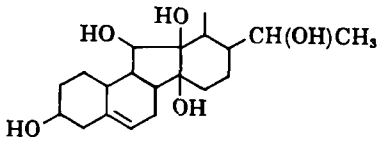
TABLE I.—PHYSICAL CONSTANTS OF SUBSTANCE *D*, *β*-AMYRIN AND THEIR ESTERS

	Substance *D*, ° C.	*β*-amyrin, ° C.
M.p.	198–199	199–200
Acetate m.p.	241–243	241
Benzoate m.p.	233	236

TABLE II.—PHYSICAL CONSTANTS OF SUBSTANCE *C*, FRIEDELIN, AND THEIR DERIVATIVES

M.p.	Substance *C*, ° C.	Friedelin, ° C.
M.p.	261–263	263–263.5
Na/propanol redn. prod.	298–299	301–304
$LiAlH_4$ redn. prod.	281–282.5	278–282
Clemmensen redn. prod.	239–240	246–249
Acetate of Na/propanol redn. prod.	313–315	315–316
Acetate of $LiAlH_4$ redn. prod.	265–267	275–276

A similar plant, *Sarcostemma australe*, thought to be responsible for stock losses in Queensland (6), has been investigated by Earl and Doherty (7) who isolated *α*- and *β*-amyrin from it. A saponin also extracted from *Sarcostemma australe* was shown to be a glycoside of an aglycone containing benzoyl and cinnamoyl groups (8). Cornforth and Earl called the hydrolysis product of the saponin "sarcostin." Reichstein, *et al.*, who obtained sarcostin from *Asclepias giancophylla* (9) and from the roots of *Pachycarpus lineolatus* (10), proposed a C-nor-D-homo-skeleton for sarcostin which enabled Cornforth (11) to propose the following structure for this substance



RESULTS AND DISCUSSION

Sarcostemma viminale, collected in midwinter from the Loskop dam area, was minced and oven-dried before extracting with benzene. The extract was evaporated to dryness, and after redissolving the residue in the least possible volume of benzene it was chromatographed on a basic alumina column. A waxy substance *A*, an amorphous solid *B*, and two crystalline substances *C* and *D* were obtained.

Elementary analysis and micro-Rast molecular weight determinations gave the formula $C_{30}H_{50}O$, for both the crystalline substances *C* and *D*. From this formula it seemed probable that both substances belong to the triterpene group.

The infrared absorption curve of *D* has a broad band at 3333 cm.$^{-1}$ indicating the presence of an associated hydroxyl band (12); further bands at 1035 cm.$^{-1}$ and 996 cm.$^{-1}$ as well as an inflection at 1026 cm.$^{-1}$ indicated an equatorial -3-hydroxy group (13). A trisubstituted ethylenic linkage was indicated by bands at 825 cm.$^{-1}$ and 813 cm.$^{-1}$ (14). The presence of this center of unsaturation was also suggested by the pale yellow color which *D* gave with tetranitromethane. The acetate and

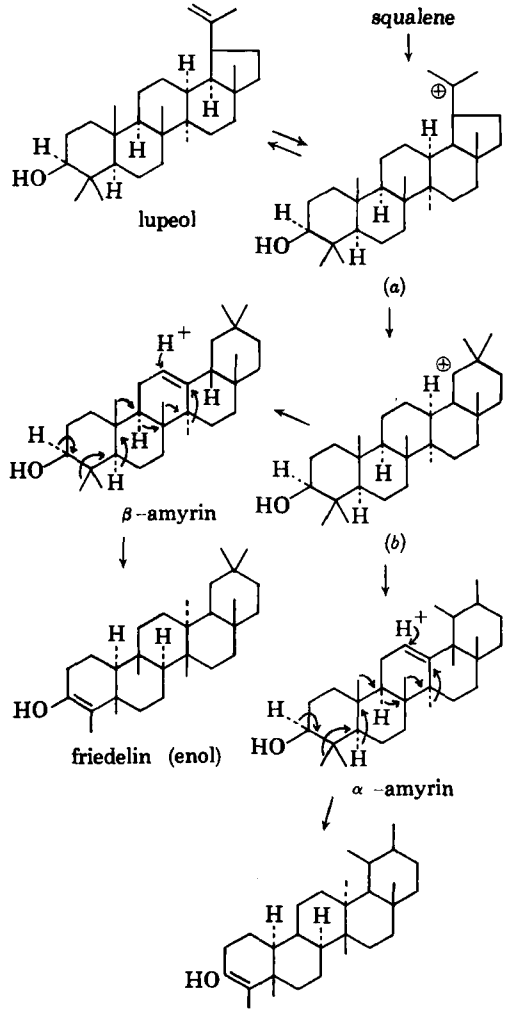


Figure 1.

TABLE III.—ANALYSIS OF COMPOUNDS

	Rast MW.	Empirical formula	Analysis Calcd, % C	Calcd, % H	Found, % C	Found, % H
Viminalol	442	$C_{30}H_{50}O$	84.50	11.74	84.05	11.61
Substance *C*	432	$C_{30}H_{50}O$	84.50	11.74	84.20	11.67
Substance *D*	446	$C_{30}H_{50}O$	84.50	11.74	84.09	11.79
Clemmensen redn. of *C*		$C_{30}H_{52}$	87.37	12.62	86.90	12.45

benzoate of *D* were prepared and from the physical constants of *D* and its esters it was concluded that *D* was β-amyrin (see Table I).

The melting point of β-amyrin showed no depression on admixture with *D* and the infrared absorption curve of an authentic sample of β-amyrin was identical with that of *D*.

The infrared curve of *C* had a sharp intense peak at 1706 cm.$^{-1}$ indicating the presence of a six-membered-ring ketone (15); no bands indicating either hydroxyl groups or unsaturation were present. Substance *C* gave no color with tetranitromethane, suggesting a saturated structure for substance *C*.

Clemmensen reduction of *C* gave the corresponding hydrocarbon $C_{30}H_{52}$. The keto group was reduced with lithium aluminum hydride and also with sodium/propanol, to give two different alcohols. The acetates of both alcohols were prepared, and from the physical constants of these compounds it was concluded that *C* was friedelin (see Table II).

An authentic sample of friedelin gave an infrared absorption curve identical with that of substance *C* and its melting point showed no depression on admixture with *C*.

It is of interest to find both β-amyrin and friedelin in the same plant since the friedelin series of compounds can be derived from the β-amyrin series by a sequence of 1:2 shifts of methyl groups and hydrogen atoms away from ring *A* toward ring *E*.

Corey and Ursprung (16, 17) have shown this relationship to exist by converting friedelin-3β-ol into olean-13-(18)-ene using various acidic reagents to bring about the multigroup rearrangement.

The same authors suggest that a possible biosynthetic pathway to friedelin in the plant is as shown in Fig. 1. The intermediate (*a*) which is originally derived from squalene can undergo a Wagner Meerwein rearrangement to give, by way of the intermediate (*b*), either α- or β-amyrin. β-Amyrin, in turn, is converted into friedelin and theoretically α-amyrin should undergo a similar transformation to form an as yet unknown derivative of friedelin.

If this biosynthetic scheme is valid, lupeol should also be found in *Sarcostemma viminale*. We have not been able to isolate lupeol, but the amorphous acetate *B* appeared to belong to the lupeol series.

This amorphous solid, after being rechromatographed in hexane on a basic alumina column, had a melting point of 160–162° but could not be crystallized. The infrared absorption curve indicated the presence of an isopropenyl group (bands at 3069 cm.$^{-1}$, 1639 cm.$^{-1}$, and 878 cm.$^{-1}$) (18) and an acetoxy group (bands at 1730 cm.$^{-1}$ and 1247 cm.$^{-1}$) (19), as well as a trisubstituted double bond (bands at 826 cm.$^{-1}$ and 808 cm.$^{-1}$) (14).

The amorphous powder was hydrolyzed with alcoholic potash to give the corresponding alcohol, m.p. 176–178°. Elementary analysis indicated the empirical formula $C_{30}H_{50}O$ for this alcohol. Since the alcohol could not be identified with any of the known triterpene alcohols, the name viminalol has been assigned to it.

The infrared curve of viminalol has a broad band at 3322 cm.$^{-1}$ indicating an associated hydroxyl group (12), further bands due to the hydroxyl group at 1036 cm.$^{-1}$, 1024 cm.$^{-1}$, and 903 cm.$^{-1}$ indicated that the hydroxyl group was most probably situated at the C-3 position of the triterpene skeleton (13). The absorption curve also showed that the isopropenyl group and the trisubstituted double bond were still present in viminalol. The latter observation was also indicated by the pale yellow color which was obtained with tetranitromethane.

The amorphous compound *B*, which must be viminalol acetate, took up one mole of hydrogen on hydrogenation in glacial acetic acid with Adam's catalyst to yield a product melting point 172–178°, which could not be crystallized. The infrared curve of this reduction product showed that the isopropenyl group was no longer present.

It is possible that viminalol acetate may be similar in nature to scandol (20, 21), which was originally reported as a new triterpene, but which was later found, by Corey, *et al.* (22), to be a mixture of β-amyrin and lupeol.

Further work is in progress to identify viminalol and substance *B*.

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Use of Dielectric Constants in the Classification of Surfactants

By WILLIAM G. GORMAN and GARY D. HALL

The polar balance of a surfactant, as reflected by its hydrophile-lipophile balance (HLB), was found to possess a linear relationship with the log dielectric constant (log DEC) of the surfactant. From this relationship, a rapid method of classifying surfactants was presented. A correlation between the polarity of an oil and that of the required emulsifying system was shown for oil-in-water emulsions. The use of direct DEC measurements for mixed surfactants and mixed oils was presented. Correlations between the DEC system and several phases of the HLB system were shown. Possible uses, advantages, and limitations of the DEC system were discussed.

IT HAS been reported (1) that the most formidable obstacle in the selection of surfactants for any particular application is the staggering number that are available. In order to efficiently and rationally select surfactants from among this number, a suitable method of surfactant classification is required. Such a method should provide a parameter that is easily measured with reliability and reproducibility. In recent years many methods (1–12) have been proposed for the classification of surfactants. Although some of these methods have been successful in limited areas of application, only the hydrophile-lipophile balance (HLB) system (1,2,8–12) appears to have found widespread usage. As its basis for classification, the HLB system successfully utilized the long recognized concept of the balance between the polar and nonpolar portions of the surfactant molecule.

Since a balance of polar and nonpolar groupings in a molecule is essentially an expression of molecular polarity, it follows that any method of measuring molecular polarity should provide a means of surfactant classification. According to Debye (13) the word polarity has been used in different ways to express molecular properties more or less connected with the actual arrangement, or the mobility, of the charges of which molecules are supposed to consist. All definitions of polarity are based on a fundamental picture which represents a molecule as a system of electric charges. Nonuniformity in the distribution of these electric charges results in polar groups which confer upon the molecules containing them a characteristic permanent electric moment, which reveals itself in dielectric phenomena. On this basis, it appeared that the polar balance of a surfactant should be quantitatively reflected in a measurement of its dielectric constant. The exploration of this hypothesis was the object of this investigation.

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EXPERIMENTAL

The dielectric constants (DEC's) of commercial grade surfactants were determined using a Sargent model V oscillometer. The instrument was calibrated using the standard procedures as outlined in the manual of instructions (14). All of the surfactants were dried prior to use in a vacuum desiccator containing calcium chloride in order to minimize experimental error due to the presence of moisture which may be significant, especially in the more hydrophilic samples. DEC values for the liquid surfactants were determined at room temperature (25 ± 1°), using the pure samples without further treatment. Because of the low melting points of the few solid surfactants examined in this study, their DEC's were determined at the lowest temperature consistent with the maintenance of the surfactant in a liquid state.

RESULTS AND DISCUSSION

Pure Surfactants.—A comparison of the reported HLB's of a number of surfactants and their experimentally determined DEC's is presented in Table I. An apparent linear relationship between these HLB values and DEC values is shown in Fig. 1, where reported HLB was plotted *vs.* log DEC. This linear relationship lends strong support to the hypothesis that surfactant polarity as inferred by its HLB value is quantitatively expressed by its DEC value.

Surfactant Mixtures.—Besides offering a system for classifying pure surfactants, DEC values also provide a means of directly measuring the polarity of mixed surfactants. The HLB system (2) claims that the HLB values of surfactants are additive in behavior on a weight-to-weight basis and that the HLB value of surfactant mixtures may be calculated accordingly. Linear relationships of an additive nature would be expected for surfactant mixtures only if the individual surfactants behaved independently of each other and exhibited no intermolecular reactions. Since it is seldom that these conditions would actually be met, a strictly additive relationship for the HLB of surfactant mixtures would not usually be expected.

Chun and Martin (6) found negative deviations from linearity in the interfacial tension measurements of Igepal[1] mixtures which suggest positive deviations from linearity of HLB values, thus indicat-

[1] Trade name for ethylene oxide condensates of alkyl phenols marketed by Antara Chemicals.

TABLE I.—COMPARISON OF DEC AND HLB VALUES OF SURFACTANTS

No., Fig. 1.	Surfactant[a]	Chemical Composition	Reported HLB Value[b]	DEC	Log DEC
1	Arlacel 83	Sorbitan sesquioleate	3.7	3.86	0.587
2	Span 20	Sorbitan monolaurate	8.6	5.57	0.746
3	Span 40	Sorbitan monopalmitate	6.7	4.88[c]	0.688
4	Span 60	Sorbitan monostearate	4.7	4.28[c]	0.631
5	Span 80	Sorbitan monooleate	4.3	4.16	0.619
6	Span 85	Sorbitan trioleate	1.8	3.37	0.528
7	Brij 30	Polyoxyethylene lauryl ether	9.5–9.7	6.02	0.780
8	PEG 200 Monolaurate	Polyoxyethylene monolaurate	9.1	6.66	0.824
9	PEG 300 Monolaurate	Polyoxyethylene monolaurate	11.3	7.27	0.862
10	PEG 400 Monolaurate	Polyoxyethylene monolaurate	12.9–13.1	7.32	0.864
11	PEG 600 Monolaurate	Polyoxyethylene monolaurate	14.5	8.14	0.911
12	Renex 648	Polyoxyethylene alkyl aryl ether	10.0	6.57	0.818
13	Renex 688	Polyoxyethylene alkyl aryl ether	12.5	7.25	0.860
14	Renex 690	Polyoxyethylene alkyl aryl ether	13.0	7.18	0.856
15	Tween 20	Polyoxyethylene sorbitan monolaurate	16.7	9.89	0.995
16	Tween 40	Polyoxyethylene sorbitan monopalmitate	15.6	9.49	0.977
17	Tween 60	Polyoxyethylene sorbitan monostearate	14.9	8.27	0.918
18	Tween 80	Polyoxyethylene sorbitan monooleate	15.0	8.75	0.942
19	Tween 81	Polyoxyethylene sorbitan monooleate	10.0	7.50	0.875
20	Tween 85	Polyoxyethylene sorbitan trioleate	11.0	7.53	0.877

[a] All surfactants were products of Atlas Chemical Industries, Inc., except the PEG monolaurates, which were products of Kessler Chemical Co. [b] Reported values as calculated or determined (1,15,16). [c] Determined at elevated temperatures.

ing that HLB values may not be strictly additive in nature. Likewise, Maclay (17) found some positive deviations from linearity in the cloud points of Pluronic[2] mixtures and Igepal mixtures.

The nonlinearity of the DEC's of mixtures of pure binary organic systems is illustrated by the work of Miller and Maass (18). Although the same deviations are observed in the dielectric constants of mixed surfactant systems, it is possible to make direct measurements even on multicomponent surfactant mixtures. The deviations from linearity of several surfactant mixtures are shown in Fig. 2. It is interesting to note that these mixtures gave positive deviations from linearity which are in general agreement with the previously reported interfacial tension and cloud point findings for other surfactant mixtures. More importantly, however, is the fact that over a considerable portion of the range for mixtures of Tween 20/Span 20 and for Tween 80/Span 80, deviations from linearity of approximately 0.04 log DEC units (approximately 1.3 HLB units) were observed.

Surfactant Solvent Relationships.—Since dielectric constants give relative values in the same numerical system for surfactants as well as for solvents (including water and oils), it is proposed that dielectric constants, in addition to offering a means of classifying surfactants, could provide a means for relating solvents and surfactants. This feature is not offered by the presently employed systems of surfactant classification. The approximate required HLB's for oil-in-water (o/w) emulsions of various oils, empirically determined by trial and error (1,2,9,12,19) have been reported. A comparison of the required HLB for o/w emulsification of several oils with the log DEC of these oils is presented in Table II. In Fig. 3 the reported required HLB of surfactant for o/w emulsion is plotted against the log DEC of these oils. Using the relationship indicated in Fig. 1, the approxi-

[2] Trade name for ethylene oxide condensates of polypropylene glycols marketed by Wyandotte Chemicals Corp.

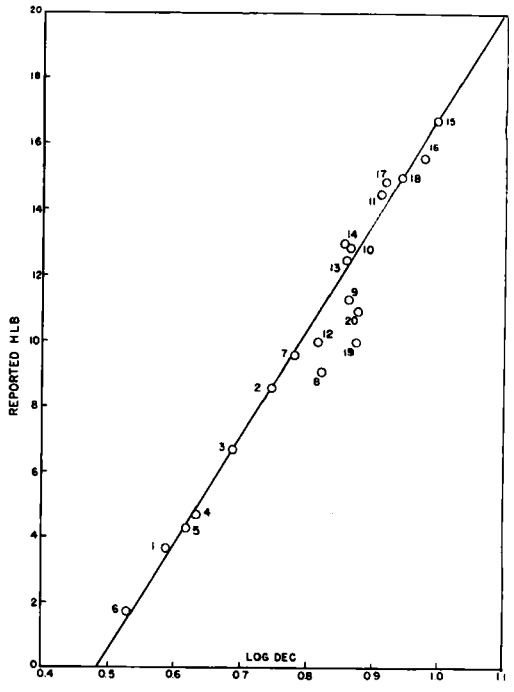

Fig. 1.—Reported HLB *versus* log DEC of pure surfactants.

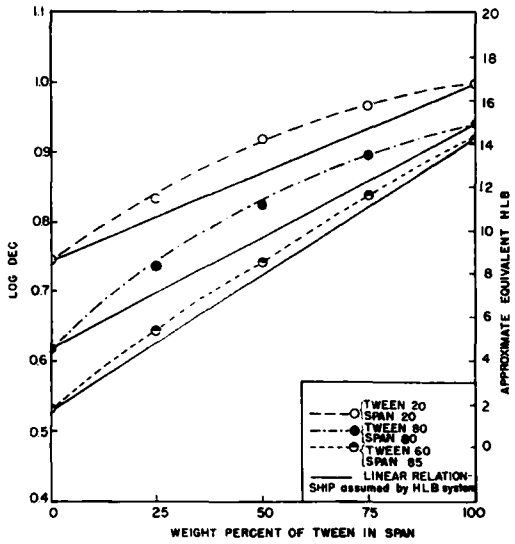

Fig. 2.—Log DEC of surfactant mixtures.

mate equivalent log DEC of surfactant is plotted as a second ordinate. Considering the fact that the required HLB values are reported as whole numbers with a range of approximately ±1 HLB unit, Fig. 3 illustrates the following interesting relationships:

1. A linear relationship exists between the log DEC of these oils and (*a*) the required HLB of surfactant for o/w emulsion and (*b*) the approximate equivalent log DEC of surfactant.

2. A proportional relationship is apparent between polarity of the oil (log DEC) and polarity (log DEC) of the surfactant required to effect o/w emulsion. The relationship suggests that for o/w emulsions, the higher the DEC of the oil, the higher would be the required DEC of the emulsifying surfactant.

3. The DEC of surfactant required for producing an o/w emulsion of any oil can be approximated from the graph by determining the DEC of the oil.

Solvent Mixtures.—The polarities of a series of oil mixtures, as indicated by their log DEC values, are reported in Fig. 4. Although these mixtures in general approximated additive or linear relationships more closely than the surfactant mixtures, there were still some deviations from linearity as shown by the kerosene-pine oil mixture, which exhibited a slight negative deviation.

The required DEC for producing o/w emulsions of oil mixtures can also be approximated, as described above, by determining the DEC of the oil mixture.

DISCUSSION

Although the data presented in this paper have been necessarily restricted to a limited number of surfactants with known HLB values, it appears that measurements of surfactant balance or polarity can be obtained for many surfactants from dielectric constant data. Such data offer distinct advantages over many of the presently employed systems since DEC's provide physical-chemical values (rather than empirical numbers) which can be duplicated experimentally with a minimum of effort and time.

In comparing DEC and HLB values, preference was given to the HLB values determined experimentally in Griffin's earlier work. It was felt that although the experimental method of determining HLB is tedious and has certain inherent weaknesses, the resulting HLB values are more significant than those calculated from the equations given in the literature (1,8,11). This seems reasonable when we consider that these equations are approximations which appear to have been empirically derived from data based on experimentally determined HLB values.

Table II.—Comparison of DEC of Various Oils and the Reported HLB Required for Producing O/W Emulsions

No., Fig. 3	Oil	DEC	Log DEC	Required HLB for o/w Emulsion[a]	HLB Ref.
1	Methyl salicylate	9.00	0.954	14	12
2	Dimethyl phthalate	8.51	0.930	15	12
3	Tricresyl phosphate	6.90	0.839	13	12
4	Lauryl alcohol	6.50	0.813	13	12
5	Castor oil	4.67	0.669	12	12
6	Pine oil	4.38	0.642	13	12
7	Isopropyl palmitate	3.15	0.498	10	12
8	Butyl stearate	3.14	0.497	11	12
	Cottonseed oil[b]	3.10	0.491	10	9
				12	12
				7.5	1, 2
9	Silicone oil	2.2–2.8	0.342–0.447	10.5	2
				11	12
10	Oleic acid	2.46	0.391	11	12
11	Toluene	2.38	0.377	9	19
12	Xylene	2.30	0.362	10	12
13	Carbon tetrachloride	2.24	0.350	9	12
14	Kerosene	2.10	0.322	12	12
				12.5	2
	Mineral oil[b]	2.10	0.322	10–12	1
				10–13	12
				10–10.5	2
				12	9

[a] Reported estimate of reliability ±1 HLB unit. [b] These oils have been omitted from Fig. 3 since there appears to be considerable variation in the reported required HLB values.

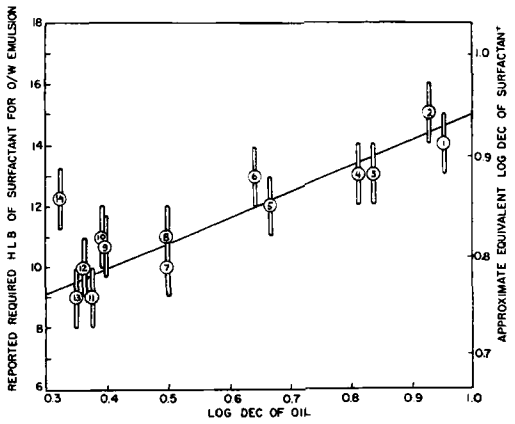

Fig. 3.—Reported required HLB of surfactant for o/w emulsion *versus* log DEC of oil.

The DEC values obtained for some solid surfactants containing large polyethylene oxide chains (usually >10 moles) were frequently lower than anticipated by their reported HLB values. This is probably due to the fact that DEC values of the pure surfactants are representative of the polar balance of the surfactant molecule alone without the influence of an aqueous or solvent environment. Since Maclay (17) reports that the high water solubility of the polyethylene oxide chain is due to hydrogen bonding between the solvent and the ether oxygen atoms in the chain, such interactions might not be apparent from DEC measurements in a solvent free environment. It is probable, however, that the extent of such solvent interactions could be determined from DEC measurements of the pure compounds and of solutions of these compounds in the solvents in question.

Since the "required HLB" of an emulsion is usually determined using a particular pair of surfactants before other groups of surfactants are considered, any deviation of the actual HLB from its theoretical value could be misleading. The positive deviations from linearity observed in the DEC values of surfactant mixtures suggest that the actual HLB's frequently deviate from the theoretical values assumed for such mixtures. These deviations were apparent whether DEC's or log DEC's were plotted *vs.* concentration, expressed in terms of % w/w, % v/v, or mole fractions. The use of DEC's could be extremely useful in the study of such mixed surfactant systems.

Expressed in terms of dielectric constants, most surfactants would have a value in the range of 3–13. However, it should be remembered that a surfactant must also have certain other properties. The molecule of a surfactant must contain one portion which is strongly attracted by the first phase or excluded by the second and another portion which should be excluded by the first phase or attracted by the second. For most systems this usually requires a lipophilic portion of substantial molecular size. Griffin (8) states that compounds should have molecular weights of at least 200 to be surfactants, while materials with molecular weights below 200 in the HLB (DEC) range of surfactants are often some of our best solvents.

Although this present investigation was intended mainly to introduce the concept and application of DEC measurements in the areas of surfactant classification and usage, there are many related applications which have not been covered in this study. Besides the general correlation of DEC values with HLB values, there also appear to be some correlations of DEC values with cloud point data, interfacial tension measurements, surface tension measurements, foaming properties, the solubilization of liquids and solids in other liquids, and critical micelle concentrations of some surfactants.

SUMMARY

1. The polar balance of a surfactant, as reflected by its hydrophile-lipophile balance (HLB), was found to possess a linear relationship with the log dielectric constant (log DEC) of the surfactant.

2. A rapid method of surfactant classification based on dielectric constants has been presented.

3. The use of this system for the direct measurement of surfactant mixtures was shown. Positive deviations from linearity were observed on the mixtures studied.

4. The use of the DEC system for the measurement of several oil mixtures and a range of polar and nonpolar solvents was described.

5. In the preparation of oil-in-water emulsions, a correlation between the DEC of the oil and that of a required surfactant system has been demonstrated. It was shown that the more polar the oil the more polar the emulsifying system required.

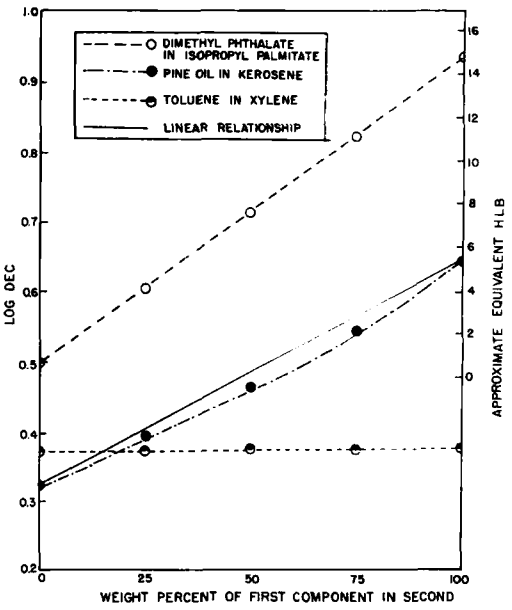

Fig. 4.—Log DEC of oil mixtures.

6. General agreement between phases of the DEC system and the HLB system was noted.

7. The DEC system was presented as offering values which can be measured directly and which can be reproduced in any laboratory by the use of established procedures.

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Antitussive Activity of a Series of Dialkylaminodiphenylbutanol Esters

By JOSEPH A. MILLER, JR., E. BROWN ROBBINS, and DONALD B. MEYERS†

The effect of structural modifications upon the antitussive activity of a series of dialkylaminodiphenylbutanol esters was examined. In an attempt to investigate a possible correlation of pharmacological activities, the compounds were also evaluated for their spasmogenic effect upon the intestine and their analgesic activity. Antitussive activity was determined in guinea pigs by means of SO_2-induced cough. Spasmogenic activity was determined by inserting a balloon into the duodenum of anesthetized dogs. Analgesic activity was determined by the rat-tail heat method. The optimum antitussive structure tested within this series was *α-dl*-2-propionoxy-1,2-diphenyl-3-methyl-4-dimethylaminobutane hydrochloride. The optimum analgesic structure tested in this series was the 2-acetoxy analog of the same compound. Some degree of correlation was shown among the activities, although exceptions were noted.

THE PRIMARY purpose of this study was to investigate the influence of certain structural modifications upon the antitussive activity of dialkylaminodiphenylbutanol esters. Since it had been observed that these compounds exhibited analgesic and spasmogenic activities, the possibility of correlations among these activities also was investigated. The antitussive evaluation was made using a new method which will be described in detail.

The compounds chosen for this study were from a series of dialkylaminodiphenylbutanol esters prepared by Pohland and Sullivan (1) of The Lilly Research Laboratories. All of these compounds were racemic mixtures, and were synthesized by the same general procedure from their corresponding ketones. The compounds used in this study are listed in Table I with their respective melting points.

EXPERIMENTAL

Antitussive Testing Method.—Many problems are associated with the evaluation of antitussive agents and a variety of methods have been employed in an attempt to overcome these difficulties. The major problem is in evoking a reproducible cough. This reproducible cough must be responsive to known antitussive agents, and the method must be sensitive enough to discriminate among varying doses of these compounds. Often this discrimination is not good and results in a flat dosage-response relationship. The numerous disadvantages and problems of most methods led to the development of the following procedure which was used to determine the antitussive activity of the diphenylbutanol esters.

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Accepted for publication September 14, 1962.
Abstracted from a thesis submitted by Joseph A. Miller, Jr., to the Graduate School of Butler University in partial fulfillment of the requirements for the degree of Master of Science.
The authors wish to thank Dr. K. K. Chen, director of the Pharmacology Division, and Dr. F. G. Henderson, head of the Department of Pharmacodynamics, Eli Lilly and Co., for their cooperation.
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Presented to The American Society for Pharmacology and Experimental Therapeutics, Rochester, N. Y., meeting, August 1961.
† Present address: Department of Pharmacology, College of Pharmacy, University of Texas, Austin.

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TABLE I.—4-DIMETHYLAMINO-1,2-DIPHENYL-2-BUTANOL ESTERS

Compound	M.p.,°C.
dl-2-Acetoxy-1,2-diphenyl-4-dimethylaminobutane HCl	227–228
α-*dl*-2-Acetoxy-1,2-diphenyl-3-methyl-4-dimethylaminobutane HCl	187–188
α-*dl*-2-Acetoxy-1,2-diphenyl-4-dimethylaminopentane HCl	210–211
α-*dl*-2-Acetoxy-1,2-diphenyl-3-methyl-4-pyrrolidinobutane HCl	202–203
α-*dl*-2-Acetoxy-1,2-diphenyl-3-methyl-4-piperidinobutane HCl	215–216
α-*dl*-2-Acetoxy-1,2-diphenyl-3-methyl-4-morpholinobutane HCl	195–196
dl-2-Propionoxy-1,2-diphenyl-4-dimethylaminobutane HCl	188–189
α-*dl*-2-Propionoxy-1,2-diphenyl-3-methyl-4-dimethylaminobutane HCl	170–171
α-*dl*-2-Propionoxy-1,2-diphenyl-4-dimethylaminopentane HCl	210–211
α-*dl*-2-Propionoxy-1,2-diphenyl-3-methyl-4-pyrrolidinobutane HCl	196–197
α-*dl*-2-Propionoxy-1,2-diphenyl-3-methyl-4-piperidinobutane HCl	191–192
α-*dl*-2-Propionoxy-1,2-diphenyl-3-methyl-4-morpholinobutane HCl	190–191

Spotted male guinea pigs weighing 250–400 Gm. were used as test animals. The guinea pigs were restrained in a plastic stock that permitted a face mask to fit flush against the front. The face mask was made by cutting off the bottom of a 6 cm. diam. plastic bottle. The neck of the bottle was fitted with a rubber stopper that had a hole bored in it to accommodate a syringe tip. The cough-producing stimulus was sulfur dioxide obtained from a cylinder. No pressure gauge was necessary, but an estimate of the gas flow was made by allowing the gas to bubble in a beaker of water in an exhaust hood. The gas was passed from the cylinder through a suction flask which served as a water trap for any water which might be siphoned into the tubing when the cylinder valve was closed. This was necessary because the sulfur dioxide within the tubing dissolved in water, resulting in a decreased pressure within the tubing. The apparatus is illustrated in Fig. 1.

Five milliliters of the gas was diluted to 10 ml. with air by means of a 30-ml. syringe, and the 10 ml. of diluted sulfur dioxide was expelled into the plastic mask held over the guinea pig's head. After the gas was administered to the guinea pigs, they were released and observed for cough frequency within the next two minutes.

A responsive colony of guinea pigs was first established by beginning with 3 ml. of gas diluted to 10, then 4 ml. diluted to 10 the next time, and finally 5 ml. diluted to 10 ml. By the third control run, nonresponders were discarded as well as those pigs on subsequent tests that did not respond after having received no treatment.

All assays were of a cross-over design in which each pig received every treatment in a randomized order. All drugs were administered subcutaneously and there were approximately ten pigs used at each dose level. There was at least one day's rest between treatments. The mean number of coughs per pig on a treatment was compared with the mean control response, and the per cent cough depression was calculated by dividing the control value into the difference between treatment and control.

Drugs were evaluated on the basis of their ED_{50}'s. This may be defined as that dose necessary to produce a 50% depression of cough. The ED_{50}'s and their standard errors were estimated by the method of Bliss (2) and are shown in Table II. Although the ED_{50} values were estimated on the basis of individual slopes, when a comparison within the series was to be made it was more appropriate to use a common slope to arrive at an estimate of relative potency. The adjusted ED_{50} of a drug was related to the adjusted ED_{50} of the reference standard.

In all methods of cough stimulation in which the recording is done by measuring the force of expired air, the possibility of false responses must be kept in mind. True cough responses may be confused with gasps, sneezes, and various contractions of the abdominal musculature without the total integrated

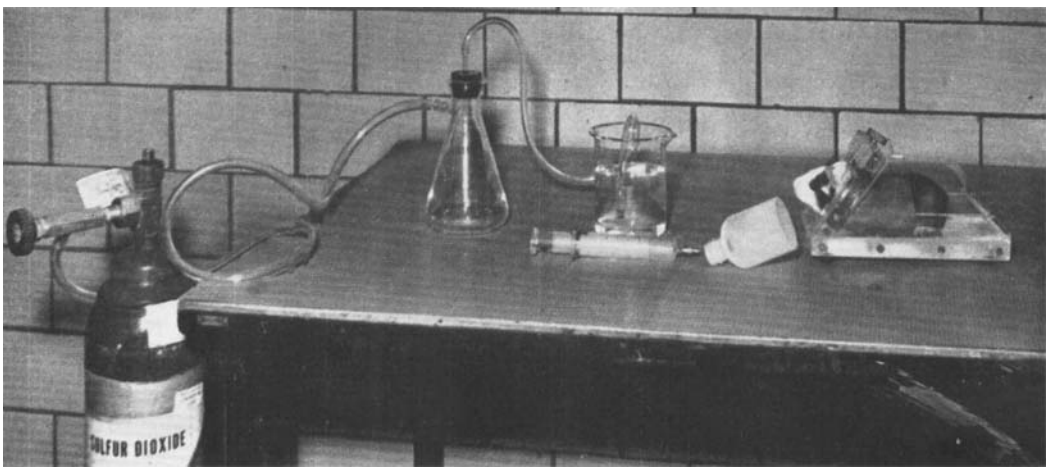


Fig. 1.—The antitussive screening apparatus. The apparatus has since been moved to a closed hood as a precaution against the sulfur dioxide fumes.

cough reflex occurring. This confusion was avoided by recording a response that was seen and heard.

Many electrical methods, due to the necessary surgery, involve acute experiments and the use of anesthetics. Due to their depressant effect on neural conduction, there is the possibility that these anesthetics as well as nonantitussive compounds with anesthetic properties will influence the results.

To avoid inconsistencies in cough response, mechanical methods of cough stimulation must be well controlled so that the stimulus is always applied equally and to the same area. This would be difficult enough to reproduce within the same laboratory using the same animal, but much more so in different laboratories.

None of these problems are encountered with the method described; the simplicity of the method eliminates many variables, and the equipment required is at a minimum. The variance in response due to animals was quite high, but error was greatly reduced by designing cross-over studies in which each animal served as his own control.

In an attempt to criticize and establish confidence in this method, those compounds which would be suspected to yield false positive results were administered, as well as known antitussive agents. Subtoxic doses of phenaglycodol,[1] a central synaptic depressant, secobarbital,[2] a barbiturate, and procaine hydrochloride, a local anesthetic, produced no depression of cough. No significant difference was shown between control (no treatment) and placebo (0.9% saline) when tested on a blind basis. This indicated that the effect of a saline injection was negligible and also showed the reproducibility of the control values in the same animals. On the other hand, morphine sulfate, codeine phosphate, and dextromethorphan hydrobromide[3] produced results (Table II) consistent with clinical findings.

Spasmogenic Testing Method.—Mongrel dogs, 6–12 Kg., anesthetized with an intravenous dose of 140 mg./Kg. phenobarbital, were used as test animals. A tracheal cannula was attached through a two-way valve to a volume-flow gas meter which measured inspired air. The carotid artery was cannulated and connected to a mercury manometer for blood pressure recording. An incision was made in the abdomen to expose the stomach. A cannula of No. PE 350 polyethylene tubing with a small balloon approximately $2^1/_2$ in. long tied around the end was inserted through an incision in the fundus of the stomach. This was passed through the pylorus and down 6 to 8 in. into the duodenum. A purse-string suture was made around the stomach incision to retard bleeding, and the abdominal incision was closed with wound clips. The balloon was inflated with approximately 6 ml. of air and attached to a tambour to record intestinal motility. All injections were made into the exposed femoral vein. A kymograph tracing of duodenal activity, blood pressure, and respiration is shown in Fig. 2.

Analgesic Testing Method.—Female, albino, Harlan strain rats weighing 70–80 Gm. were used as test animals. The method was a modification (3) of that used by Davies and co-workers (4). Mean reaction times of treated rats were compared with mean control values obtained at the same time to arrive at a mean increase in reaction time for each treatment group. A mean increase in reaction time of 1.5 seconds per rat ($ED_{1.5}$) was arbitrarily established as a basis for comparing different drugs. This represented a significant rise in threshold response. Dosage-response curves were calculated for each drug, and using individual slopes, the dose necessary to obtain an increase in reaction time of 1.5 seconds was computed. These figures are to be found in Table II.

The error was estimated by the method used for determining regression coefficients found in most standard statistical texts (5). The error of log dose x was estimated for the special case where y equals 1.5 seconds.

As was done in the case of antitussive evaluations, relative potency figures were estimated on the basis of a common slope and the adjusted $ED_{1.5}$ values were compared with the adjusted $ED_{1.5}$ of the reference standard.

RESULTS

The results of the antitussive, analgesic, and spasmogenic evaluations are summarized in Table II. The minimal toxic dose refers to the dose that produced the first observable signs of toxicity. These consisted of either sedation or tremors. The spasmogenic dose refers to the lowest dose that produced any increase in activity of the duodenum. Explanations of the remaining columns have been given under the appropriate testing methods.

Dotted spaces indicate that no significant effect was seen at subtoxic doses. A dotted space will also be noted after the figure 54.2 ± 26.7 on line 9, under "Analgesic Activity." Although this compound was of a low-order analgesic potency, an estimate of the $ED_{1.5}$ was not impossible. However, due to its poor dosage-response relationship and flat slope, it was not included in the calculation of relative potency. It was felt that inclusion would flatten the common slope of the remaining active compounds, resulting in distorted estimates of relative potency.

DISCUSSION

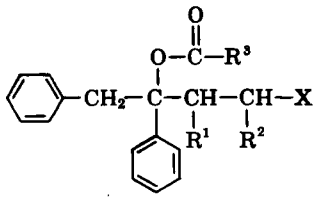
In the following discussion of structure-activity relationships all remarks will be limited to those twelve compounds that were reported in this paper. It must also be emphasized that these were racemic mixtures and that observations and conclusions apply only to these mixtures and not to their optical isomers. From previous reports, it might be expected that there would be a division of activity in the isomers upon resolving the racemate. In the case of the α-*dl*-2-propionoxy-1,2-diphenyl-3-methyl-4-dimethylaminobutane analog (*dl*-propoxyphene), it was demonstrated (6) that all of the analgesic activity resided in the dextrorotatory isomer. Also, this compound was shown to be unique in that the nonanalgesic isomer (*l*) possessed greater antitussive activity than the *d* isomer or the racemic mixture.

No structure-activity study should be considered complete without a study of the optical isomers within that series, and further work in this area should provide additional valuable data.

Antitussive Activity.—In the acetoxy derivatives,

[1] Marketed as Ultran by Eli Lilly and Co.
[2] Marketed as Seconal Sodium by Eli Lilly and Co.
[3] Marketed as Romilar, kindly supplied by Hoffmann-LaRoche, Inc.

TABLE II.—ANTITUSSIVE, ANALGESIC, AND SPASMOGENIC TEST RESULTS



				Antitussive Activity Guinea Pigs, s.c.				Analgesic Activity Rats, s.c.			Intestinal Activity Dogs, i.v. Spasmogenic
R¹	R²	R³	X	ED_{50} ± S.E., mg./Kg.	Relative Potency, % Morphine	Minimal Toxic Dose, mg./Kg.	Peak Effect, minutes	$ED_{1.5}$ ± S.E., mg./Kg.	Relative Potency, %, Morphine	Minimal Toxic Dose, mg./Kg.	Dose, mg./Kg.
H	H	CH_3	$N(CH_3)_2$	25.5 ± 5.0	50	80	60	...	...	160	3.2
CH_3	H	CH_3	$N(CH_3)_2$	8.5 ± 1.8	138	20	30	3.3 ± 0.5	23	12	0.04
H	CH_3	CH_3	$N(CH_3)_2$	12.5 ± 7.6	90	40	90	14.4 ± 1.9	4	40	0.8
CH_3	H	CH_3		5.8 ± 1.2	247	20	60	5.0 ± 1.0	14	20	0.05
CH_3	H	CH_3		23.9 ± 7.5	46	40	30	8.5 ± 1.3	7	20	0.08
CH_3	H	CH_3		...	...	40	30	...	...	320	3.2
H	H	C_2H_5	$N(CH_3)_2$	7.6 ± 3.5	138	160	60	...	...	320	0.1
CH_3	H	C_2H_5	$N(CH_3)_2$	3.5 ± 1.0	292	40	30	6.6 ± 0.6	9	20	0.2
H	CH_3	C_2H_5	$N(CH_3)_2$	23.9 ± 9.7	48	160	60	54.2 ± 26.7	...	160	1.6
CH_3	H	C_2H_5		9.8 ± 1.4	119	80	30	9.6 ± 2.2	7	40	0.4
CH_3	H	C_2H_5		23.3 ± 6.7	53	80	30	...	...	320	3.2
CH_3	H	C_2H_5		...	..	80	30	...	...	640	3.2
Morphine sulfate				9.8 ± 2.1	100	16	30	0.8 ± 0.2	100	4	0.002
Codeine sulfate				25.4 ± 2.9	30	60	30	2.7 ± 0.2	123	20	0.1
Dextromethorphan hydrobromide				18.8 ± 5.8	31	30	30	...	...	64	0.2

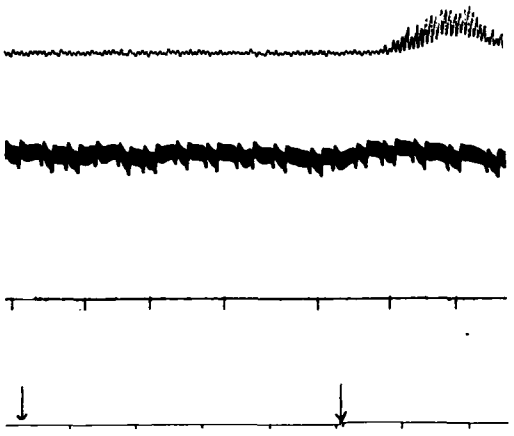


Fig. 2.—The effect of .025 mg./Kg. followed by .05 mg./Kg., i.v. of α-*dl*-2-acetoxy-1,2-diphenyl-3-methyl-4-pyrrolidinobutane hydrochloride on the duodenum of an 8.8 Kg. female dog, anesthetized with 140 mg./Kg. sodium phenobarbital i.v. Top line, duodenal activity; second line, blood pressure; third line, respiratory volume in liters; fourth line, time in minutes.

the compound unsubstituted at R^1 and R^2 possessed weak antitussive activity. Substitution at R^2 with a methyl group enhanced activity, but substitution at R^1 was more effective. The activity of the R^1 methyl-substituted compound was further enhanced by the substitution of a pyrrolidine group for the dimethylamine group. A piperidine substitution at *x* resulted in decreased activity and the morpholine-substituted analog was void of antitussive activity.

In the propionoxy derivatives, results obtained by substituting a methyl group at R^1 and R^2 were inconsistent with the acetoxy analogs. Although both R^1 methyl-substituted analogs were more potent than their corresponding unsubstituted or R^2 methyl-substituted analogs, the R^2 methyl-substituted propionoxy analog was less potent than its unsubstituted (R^1 and R^2 = H) analogs. This was not true in the acetoxy derivative. A pyrrolidine substituent at *x* did not enhance potency as was the case in its acetoxy analog. The piperidine analog was less potent than the acetoxy analog, and in both cases the morpholine analogs were inactive.

From the data it was concluded that the most effective antitussive structure among these compounds was represented by the R^1 methyl-substituted, propionoxy, dimethylamine analog. Pharmacological studies of this compound have been reported previously in the literature (3).

Spasmogenic Activity.—The spasmogenic data on the dog intestine were quite difficult to correlate. The active compounds in the series fell within the range of 0.04 to 0.4 mg./Kg. i.v., which is consistent with other antitussives and analgesics except morphine. The acetoxy derivatives were more active than their corresponding propionoxy analogs, and those compounds which showed weak or no antitussive and analgesic activity were consistently less active in their intestinal effect. If a compound had shown either antitussive or analgesic activity, it was also active upon the intestine. In this respect, it would be concluded that spasmogenic activity paralleled antitussive or analgesic potency, but not in any direct relationship.

Analgesic Activity.—In the compounds tested, it was observed that the acetoxy derivatives were consistently more potent than their propionoxy analogs. This relationship also held true with respect to toxicity resulting in no therapeutic advantage.

The unsubstituted (R^1 and R^2 = H) dimethylaminobutane acetoxy analog produced no analgesia. Substitution with a methyl group at R^2 produced an analgesically active compound and this activity was enhanced by shifting the methyl group to the R^1 position. The substitution of a pyrrolidine group for the dimethylamine moiety resulted in a slight loss of analgesic activity. Activity was further reduced by the substitution of a piperidine group at *x*. The morpholine analog was void of analgesic activity.

In the slightly less active propionoxy analogs, the R^1 methyl-substituted compound was also the most active of the dimethylamine analogs. A progressive loss in activity resulted from shifting the R^1-methyl group to the R^2 position and finally removing it. The effects of the pyrrolidine substitution at *x* were consistent with the acetoxy analog, but the piperidine substitution resulted in a total loss of activity. The morpholine analogs of the acetoxy and propionoxy derivatives were consistently inactive. The ratio of the $ED_{1.5}$ to the minimal toxic dose (therapeutic index) was roughly the same throughout the series.

It was concluded that among these compounds, the acetoxy derivative with a methyl group in the R^1 position and a dimethylamine moiety at *x* represented the optimum analgesic structure.

Correlation.—Only one compound (α-*dl*-2-propionoxy-1,2-diphenyl-4-dimethylaminobutane hydrochloride) exhibited good antitussive activity without analgesic activity. The remaining compounds in which antitussive activity was demonstrated also exhibited analgesic activity. There was only one compound of good analgesic potency which exhibited low antitussive potency (α-*dl*-2-acetoxy-1,2-diphenyl-3-methyl-4-piperidinobutane hydrochloride). All compounds which were active upon the intestine were also active as analgesics and/or antitussives. These observations are suggestive of some degree of correlation between analgesic and antitussive potency, while a strong correlation was shown between antitussive activity and spasmogenic activity although not quantitatively so.

SUMMARY

1. A series of dialkylaminodiphenylbutanol esters was tested for antitussive activity, and this activity was compared with their analgesic potencies and effect upon the intestine.

2. A new method for evaluating antitussive activity was described.

3. The optimum antitussive structure within this series tested was represented by α-*dl*-2-propionoxy - 1,2 - diphenyl - 3 - methyl - 4-dimethylaminobutane hydrochloride.

4. The optimum analgesic structure within this series tested was represented by α-*dl*-2-acetoxy - 1,2 - diphenyl - 3 - methyl - 4 - dimethylaminobutane hydrochloride.

5. Some degree of correlation was shown among the three activities, although exceptions were noted.

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Chlorpromazine and Dextro-amphetamine as Antidotes in Acute Antihistaminic Toxicity in Rats

By I. H. MAEL† and J. F. BESTER

Acutely toxic oral doses of a number of antihistaminics were determined in adult white rats of both sexes. Toxic symptoms included convulsive seizures which lasted some minutes and were periodically repeated, followed by generalized central depression, terminating in unconsciousness and death. Chlorpromazine and dextro-amphetamine were administered in that order, intraperitoneally, in varying quantities and at varying time intervals. It was found that, if chlorpromazine was administered at the onset of convulsions and dextro-amphetamine at the first signs of depression, the recovery rate of animals was quite high.

THERAPEUTICALLY useful antihistaminics were developed well over 20 years ago, and for most of that time their acutely toxic capabilities have been recognized. Just as the various antihistaminics vary in their potencies, so they vary in the frequency and severity of their toxic manifestations. Such variations are largely quantitative, however; and when they occur, the symptoms of acute toxicity are quite similar regardless of the drug used. Typically, drowsiness followed by nervousness, tremors, muscle twitching, delirium, and convulsions are observed along with respiratory depression and cyanosis and followed by unconsciousness and death (1).

Despite the fact that repeated efforts have been made to find satisfactory methods of treatment of acute poisoning with the antihistaminic drugs, methods of treatment continue to be difficult and not overly successful. The need for uniformly adequate antidoting has not lessened with the years; acute antihistaminic poisoning is not uncommon. Each month, for example, several such intoxications are reported in Arizona, totaling 27 for the year 1961 (2). The Poison Information Center in Los Angeles in the first 6 months of 1961 reported 42 acute poisonings from antihistaminics as such, plus an additional 65 due to antihistaminic-containing nonbarbiturate sedatives (3).

The difficulty in preventing poisoning with these drugs and in coping with them when they arise is complicated by the fact that dose effect relationships are inconsistent. For example, the oral LD_{50} of diphenhydramine in rats has been reported as 500 mg./Kg. (4), and of pyrilamine hydrochloride, subcutaneously in rats, as 150 mg./Kg. (5). Yet 400 mg. of diphenhydramine and 1.3 Gm. of pyrilamine, respectively, caused the deaths of two 2-year-old children (1). Connolloy reported serious intoxication of a $5^1/_2$-year-old child following ingestion of 12 mg. of chlorpheniramine (6).

Because antihistaminics typically cause toxic symptoms of mixed characteristics, single antidotes offer little hope of success. Various drugs and drug combinations have been tried and reported. These include histamine, atropine plus epinephrine, phenobarbital, caffeine, dextro-amphetamine (7), ether (8), phenobarbital plus caffeine and ephedrine (9). Results were generally unsatisfactory.

Chlorpromazine reportedly has shown the ability to cause a definite decrease in motor activity without evidence of hypnosis (10). Dextro-amphetamine has long been recognized as an

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TABLE I.– ESTABLISHMENT OF DOSAGE AND ANTIDOTAL SCHEDULES

No. of Rats	Drugs Given	Dosage, mg./Kg.	When Given	Results
5	Diphenhydramine	250	...	Recurring epileptiform convulsions. All dead within 2 hours.
5	Chlorpromazine	50	...	Depression; three deaths.
5	Diphenhydramine Chlorpromazine	250 50	Simultaneously	Severe depression; four deaths.
5	Diphenhydramine	100	...	Excitation only; no convulsions.
5	Diphenhydramine Chlorpromazine	100 25	Simultaneously	Marked depression only.
5	Diphenhydramine Chlorpromazine	250 25	Simultaneously	Depression and death of all.
5	Diphenhydramine Chlorpromazine	250 15	Simultaneously	Depression and death of all.
5	Diphenhydramine Chlorpromazine	250 10	Simultaneously	Depression and death of all.
5	Diphenhydramine Dextro-amphetamine	250 0.15	Simultaneously	Convulsions; all dead in 2 hours.
5	Diphenhydramine Chlorpromazine	250 10	Simultaneously	Depression; all dead in 24 hours.
...	Dextro-amphetamine	0.75	Upon onset of depression	...
5	Diphenhydramine Chlorpromazine Dextro-amphetamine	250 10 1.25	... Simultaneously upon onset of symptoms	No convulsions, but excitation. Later depression with three deaths.
10	Diphenhydramine	250	...	All symptoms well controlled.
...	Chlorpromazine	10	Upon onset of convulsion	...
...	Dextro-amphetamine	0.625	Upon onset of depression	...
...	Dextro-amphetamine	0.3	As needed	...

analeptic. It therefore appeared logical to test their combined abilities in controlling the acute toxic manifestations of antihistaminic agents.

EXPERIMENTAL

White adult Wistar strain rats of both sexes were employed in the study. They were deprived of food and water for 24 hours prior to use. Antihistaminic drug doses were administered in aqueous solution by stomach tube. Concentration of solutions was adjusted so that all doses were of the same volume. Antidotal drugs were administered intraperitoneally, and again volumes were kept constant.

Diphenhydramine was used initially because its action and side effects seemed most typically representative of the group (11).

After antidoting, the animals were kept under observation for 72 hours.

Initial experimental procedures were designed to establish appropriate doses, dosage schedules, and sequence of drug administration. The results of this testing are shown in Table I.

Symptoms exhibited by those animals receiving only diphenhydramine followed closely the pattern described for humans. The time between drug administration and first symptoms varied from 8 minutes to 65 minutes. Excitation increased, particularly to such stimuli as noise and touch. Respiratory depression developed. Spontaneous movements increased, but gait became unsteady. A Straub reaction occurred in some animals. Muscle twitching steadily increased in intensity until full epileptiform convulsions ensued. Cyanosis was quite evident. Short periods of collapse separated convulsive episodes. Unconsciousness, accompanied by decreasing convulsions and increasing cyanosis, led ultimately to death.

Those animals receiving diphenhydramine plus chlorpromazine showed only the signs of depression; those receiving diphenhydramine plus dextro-amphetamine developed the signs of excitation more rapidly and more severely.

Because the last procedure shown in Table I appeared to be most effective, a total of 32 additional animals were treated by this routine. Diphenhydramine (250 mg./Kg.), was administered orally. As soon as convulsions became evident, chlorpromazine (10 mg./Kg.), was given intraperitoneally and as depression developed, dextro-amphetamine (0.625 mg./Kg.), was given intraperitoneally. If depression continued or returned, further doses of dextro-amphetamine (0.3 mg./Kg.), were given. In no instance was it necessary to administer more than three such additional doses.

DISCUSSION

All 32 animals showed the initial symptoms of toxicity, but only 22 developed convulsive seizures and only these were treated. The remaining ten animals recovered without treatment. Of the 22 treated, three failed to respond to chlorpromazine

and died during convulsions. Of the 19 whose convulsions ceased following chlorpromazine administration only one died during the ensuing depression while 18 recovered. Thus, of a total of 42 animals receiving 250 mg./Kg. of diphenhydramine, 32 developed symptoms requiring treatment and 28 of these recovered.

Limited studies of the effectiveness of this regimen in combating acutely toxic doses of several other antihistaminics were conducted. The protocol was unchanged, except that challenging doses of antihistaminics were altered to parallel potency. The results are reported below.

Tripelennamine.—Doses of 100, 150, 200, and 250 mg./Kg., respectively, were administered to eight rats. Chlorpromazine (4 mg./Kg. for each 100 mg./Kg. of tripelennamine), and dextro-amphetamine (0.25 mg./Kg. for each 100 mg./Kg. tripelennamine), served as antidotes. Only at the largest challenging dosage level was it necessary to give more than one dose of dextro-amphetamine. At this level one animal died. All others recovered.

Thenylpyramine.—Dosage and antidotal procedures were identical with those for tripelennamine. However, in all cases three doses of dextro-amphetamine were given. At each of the 200 and 250 mg./Kg. levels of thenylpyramine one animal died. All others recovered.

Chlorphenamine.—Challenging doses were 10, 15, 20, and 25 mg./Kg., respectively. Antidoting doses were 4 mg./Kg. of chlorpromazine and 0.25 mg./Kg. of dextro-amphetamine for each 10 mg./Kg. of chlorphenamine. A single administration of dextro-amphetamine sufficed in all cases and all animals recovered.

At the present time, these studies are continuing. Because current antidotal procedures against antihistaminic acute toxicity leave much to be desired, any improvement in method warrants further study. It is hoped that the combination of drugs herein described, or closely related drugs to be tested in our laboratory, will continue to show promise.

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Solubility of Carbon Dioxide, Krypton, and Xenon in Lipids

By SHU-YUAN YEH and RICHARD E. PETERSON

The solubility of carbon dioxide, krypton, and xenon has been measured at one atmospheric pressure and at temperatures of 25, 30, 37, and 45° in olive oil, dog fat, human fats, and rat-pooled fat. The solubility of the gases studied in the oil and fats was found to decrease as the temperatures increased. Heats and entropies of solution have been calculated from linear dependencies of logarithm of the solubility with reciprocal absolute temperature. There seems to be a linear relationship between solubility, surface tension, and viscosity of the lipids.

THE PRIMARY objective of this study is to obtain some precise data on the solubility of krypton and xenon in lipids for calculation of body fat in a living body. Carbon dioxide was also included in this study because of the paucity of such data in lipids.

The solubilities of carbon dioxide, air, oxygen, nitrogen, and hydrogen in corn oil, in both unhydrogenated and hydrogenated lard, and in cottonseed oil have been measured at various temperatures by several investigators (1–3). They found that all the gases, with the exception of carbon dioxide, are increasingly soluble in the fat as the temperature rises. The solubility of carbon dioxide in human fat, dog fat, and rat fat at 38° was reported by Nichols (4). The solubility of xenon in olive oil and other aromatic oils, and of radioactive krypton and xenon in olive oil has been measured at 20, 22, and 37° by Steinberg and Manowitz (5), and Lawrence, *et al.* (6), respectively. Steinberg and Manowitz claimed their data, which are identical with Lawrence, *et al.*'s, to be accurate within 10%. It is obvious that these data are not sufficiently precise for calculating body fat in a living body.

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EXPERIMENTAL

Materials Employed.—Research grade carbon dioxide, krypton, and xenon were purchased from Matheson Co. According to their specifications,

and died during convulsions. Of the 19 whose convulsions ceased following chlorpromazine administration only one died during the ensuing depression while 18 recovered. Thus, of a total of 42 animals receiving 250 mg./Kg. of diphenhydramine, 32 developed symptoms requiring treatment and 28 of these recovered.

Limited studies of the effectiveness of this regimen in combating acutely toxic doses of several other antihistaminics were conducted. The protocol was unchanged, except that challenging doses of antihistaminics were altered to parallel potency. The results are reported below.

Tripelennamine.—Doses of 100, 150, 200, and 250 mg./Kg., respectively, were administered to eight rats. Chlorpromazine (4 mg./Kg. for each 100 mg./Kg. of tripelennamine), and dextro-amphetamine (0.25 mg./Kg. for each 100 mg./Kg. tripelennamine), served as antidotes. Only at the largest challenging dosage level was it necessary to give more than one dose of dextro-amphetamine. At this level one animal died. All others recovered.

Thenylpyramine.—Dosage and antidotal procedures were identical with those for tripelennamine. However, in all cases three doses of dextro-amphetamine were given. At each of the 200 and 250 mg./Kg. levels of thenylpyramine one animal died. All others recovered.

Chlorphenamine.—Challenging doses were 10, 15, 20, and 25 mg./Kg., respectively. Antidoting doses were 4 mg./Kg. of chlorpromazine and 0.25 mg./Kg. of dextro-amphetamine for each 10 mg./Kg. of chlorphenamine. A single administration of dextro-amphetamine sufficed in all cases and all animals recovered.

At the present time, these studies are continuing. Because current antidotal procedures against antihistaminic acute toxicity leave much to be desired, any improvement in method warrants further study. It is hoped that the combination of drugs herein described, or closely related drugs to be tested in our laboratory, will continue to show promise.

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Solubility of Carbon Dioxide, Krypton, and Xenon in Lipids

By SHU-YUAN YEH and RICHARD E. PETERSON

The solubility of carbon dioxide, krypton, and xenon has been measured at one atmospheric pressure and at temperatures of 25, 30, 37, and 45° in olive oil, dog fat, human fats, and rat-pooled fat. The solubility of the gases studied in the oil and fats was found to decrease as the temperatures increased. Heats and entropies of solution have been calculated from linear dependencies of logarithm of the solubility with reciprocal absolute temperature. There seems to be a linear relationship between solubility, surface tension, and viscosity of the lipids.

THE PRIMARY objective of this study is to obtain some precise data on the solubility of krypton and xenon in lipids for calculation of body fat in a living body. Carbon dioxide was also included in this study because of the paucity of such data in lipids.

The solubilities of carbon dioxide, air, oxygen, nitrogen, and hydrogen in corn oil, in both unhydrogenated and hydrogenated lard, and in cottonseed oil have been measured at various temperatures by several investigators (1–3). They found that all the gases, with the exception of carbon dioxide, are increasingly soluble in the fat as the temperature rises. The solubility of carbon dioxide in human fat, dog fat, and rat fat at 38° was reported by Nichols (4). The solubility of xenon in olive oil and other aromatic oils, and of radioactive krypton and xenon in olive oil has been measured at 20, 22, and 37° by Steinberg and Manowitz (5), and Lawrence, *et al.* (6), respectively. Steinberg and Manowitz claimed their data, which are identical with Lawrence, *et al.*'s, to be accurate within 10%. It is obvious that these data are not sufficiently precise for calculating body fat in a living body.

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EXPERIMENTAL

Materials Employed.—Research grade carbon dioxide, krypton, and xenon were purchased from Matheson Co. According to their specifications,

carbon dioxide contained maximum possible impurities of 0.3% mole of nitrogen, or carbon monoxide and oxygen; krypton contained maximum possible impurities of 0.04% nitrogen and xenon; and xenon contained maximum possible impurities of 0.02% nitrogen and 0.05% krypton.

Olive oil U.S.P. was purchased from Magnus, Mabee and Raynard Co., Inc.

Dog Fat.—Dog perineal, mesenteric, omental, and other adipose fats were extracted with petroleum ether C.P. (b.p. 36–65°). The fat was stored in a screw-capped jar under refrigeration after the ether was evaporated from the fat under vacuum at about 80° for several hours.

Human Fat.—Human omental fats, obtained from two different deceased patients, were marked as human fat No. 1 and No. 2, respectively, and treated as described under "Dog Fat." The human fats No. 1 and No. 2 seemed no different, except that the human fat No. 2 appeared to have more precipitate (stearine) than No. 1 at the temperature of about 23°.

Rat Fat.—Rat retroperitoneal, mesenteric, omental fats, and hair clipped skin, which was cut in about one square inch sections, were dried at about 80° under vacuum, crushed coarsely, and finally extracted with petroleum ether in a Soxhlet extractor. The procedure, as described under "Dog Fat," was then followed.

Measurement of the Solubility of Gases in Lipids.—The apparatus used is shown in Fig. 1. *A* is a modified Geffken's gas buret (7). Burets *B* and *C* include three 20-ml. bulbs and four 5-ml. bulbs, respectively. The volume of *D* is 5 ml. and is graduated to 0.01 ml. within a 45 cm. length. The volume of the ungraduated part of the modified buret, up to stopcock *3* and the volume of the graduated parts between the marks, were calibrated with mercury at 25° and 30°. The capacity of the connecting tube, from stopcocks *3* to *7*, and above the oil surface of the differential oil manometer, was determined by measuring the gas volume to fill the evacuated tube. The whole buret was enclosed in a water jacket and kept constant in temperature within ±0.05° by circulating water from a thermostat bath, which was equipped with a heating and refrigerating unit.

L is an absorption flask, of about 50 ml. capacity, with a standard taper male joint and a mercury sealing cup on the top. The volume of the absorption flask, containing the glass sealed stirring bar, *K*, up to stopcock *6*, was determined by measuring the gas volume to fill the evacuated flask at several experimental temperatures.

R is a differential oil manometer, which was charged with Octoil-S (2-ethyl-hexyl-sebacate) pump oil (saturated with the experimental gas), and was kept with the pure experimental gas above the oil surfaces through stopcocks *4* and *5* after it was charged.

The connection between the pressure reducing valve, gas tank, and copper tube was screw-tightened and sealed with wax. All joints and stopcocks in the whole system were tested from point to point to make sure there was no leakage before an experiment was conducted. The pressure from any part could be evacuated down to 50–100 μ, as measured with the McLeod gauge.

Manipulation.—Part of the prepared fat was

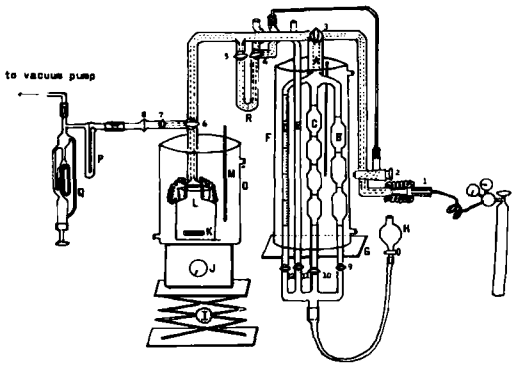

Fig. 1.—Apparatus for the determination of solubility of gases.

dried under stirring and vacuum at about 80° for about 12 hours (overnight) before it was charged to the absorption flask. All moisture or volatile materials should be evacuated under this treatment (8). The dried sample was charged to the cleaned and dried absorption flask and stirring bar by semimicro differential weighing. After the flask was assembled to the apparatus, the mercury in the gas buret was raised up to stopcock *3* and the air of the connecting system was evacuated. The gas was passed from the tank into the connecting tube and the gas buret and equilibrated with the temperature at least one hour before it was passed into the absorption flask. Water was circulated into the bath, *O*, and the oil was stirred and evacuated from the flask until no bubbles appeared. The apparatus was then disconnected from the vacuum pump. The gas pressure in the gas buret was adjusted to the ambient pressure and the initial reading was taken. Stopcocks *4* and *5* were turned off immediately after the reading was taken. Gas was passed into the absorption flask by turning stopcock *6* on and off occasionally until equilibrium was reached as indicated by the differential oil manometer for at least one-half hour. The temperature of the absorption flask was lowered as the thermoregulator was reset at a lower temperature. A series of solubilities at lower temperatures was obtained by this stepwise method.

This apparatus and technique have been tested with carbon dioxide in water. The solubility of carbon dioxide in water at 25, 30, 37, and 45° gave α = 0.7438, 0.7457; 0.6589, 0.6551; 0.5568, 0.5542; 0.4716, 0.4683, compared with the literature value (30) of 0.759, 0.665, 0.562, 0.479. The reproducibility of the data, obtained from this apparatus and technique, is within ±0.5%. It is believed that the apparatus and method described afford several advantages over other apparatus previously used. The liquid was constantly stirred in the absorption flask with a glass sealed magnet stirring rod, thus facilitating degassing as well as the saturation process and avoiding supersaturation. A considerable series of solubility data can be obtained with a single sample charged in the absorption flask. The solvent does not come in contact with mercury surfaces, thus eliminating contamination, or reaction, of the liquid with mercury. A water bath, whose temperature is easily controlled at a constant value, was used and the need for an elaborate air

thermostat was eliminated. The gas buret can be recharged for larger solubilities. It is easy to clean and dry the absorption flask and to charge the sample accurately, especially with the more viscous oil, fat, and tissue homogenates. In addition, an oil differential manometer was added to the system, thus increasing the sensitivity of the change of gas pressure within the gas buret and allowing one to easily determine whether the liquid was equilibrated with the gas or not.

The absorption coefficient, α, as defined by Bunsen (9), is given by the formula

$$\alpha = \frac{V_{o(a)}}{v}\frac{P_o}{P} = \left[\frac{V_{o(1)} + V_{o(C_1)} - V_{o(2)} - V_{o(C_2)} - V_{o(g)}}{v}\right]\frac{P_o}{(P_B - P_S)}$$

where $V_{o(a)}$, $V_{o(1)}$, $V_{o(C_1)}$, $V_{o(2)}$, $V_{o(C_2)}$, and $V_{o(g)}$ are the volumes of the gas absorbed, originally introduced in the gas buret and connecting tube, remaining in the gas buret, the connecting tube, and over the liquid surface in the absorption flask after equilibrium at standard temperature and pressure.

v is the volume of liquid at the saturation temperature, equal to the weight of liquid charged, divided by the density of the liquid at the saturation temperature. P is the partial pressure of the gas in mm. Hg. P_B is the corrected barometric pressure at the end of saturation. P_S is the vapor pressure of liquid at the experimental temperature and P_o is 760 mm. Hg.

Measurement of the Density of Lipids.—The densities of olive oil and fats were determined at 25 and 30° according to the standard procedure (10). The densities of olive oil and fats at the other temperatures studied were calculated according to the well established equation (11)

$$D_2 = D_1 - K(t_2 - t_1)$$

TABLE I.—SOLUBILITIES OF CARBON DIOXIDE IN LIPIDS

Lipid	Temp., °C.	Bunsen s Absorption Coefficient Mean	S.D.	Lit. Value	Ostwald Solubility Coefficient
Olive oil	45	1.0150	0.0015		1.1821
	37	1.1306	0.0016		1.2849
	30	1.2519	0.0016		1.3893
	25	1.3565	0.0013		1.4806
Dog fat	45	1.0122	0.0011		1.1790
	37	1.1283	0.0014	0.861[a]	1.2822
	30	1.2491	0.0015		1.3862
	25	1.3411	0.0022		1.4638
Human fat 1	45	1.0153	0.0020		1.1826
	37	1.1349	0.0018	0.848[a]	1.2897
	30	1.2471	0.0015		1.3840
	25	1.3589	0.0015		1.4833
Human fat 2	45	1.0122	0.0009		1.1790
	37	1.1254	0.0014		1.2789
	30	1.2448	0.0044		1.3815
	25	1.3376	0.0044		1.4600
Rat-pooled fat	45	0.9989	0.0041		1.1635
	37	1.1125	0.0035	0.898[a]	1.2643
	30	1.2344	0.0051		1.3700
	25	1.3363	0.0073		1.4586

[a] See reference 4.

TABLE II.—SOLUBILITIES OF KRYPTON IN LIPIDS

Lipid	Temp., °C.	Bunsen's Absorption Coefficient Mean	S.D.	Lit. Value[a]	Ostwald Solubility Coefficient
Olive oil	45	0.3844	0.0021		0.4477
	37	0.4031	0.0025	0.43[b]	0.4581
	30	0.4225	0.0025		0.4688
	25	0.4376	0.0010	0.44 (22°)[b]	0.4746
Dog fat	45	0.3853	0.0004		0.4426
	37	0.4031	0.0007		0.4581
	30	0.4225	0.0013		0.4721
	25	0.4364	0.0006		0.4764
Human fat 1	45	0.3878	0.0015		0.4516
	37	0.4071	0.0005		0.4626
	30	0.4258	0.0012		0.4725
	25	0.4412	0.0061		0.4816
Human fat 2	45	0.3875	0.0012		0.4513
	37	0.4062	0.0004		0.4617
	30	0.4247	0.0013		0.4713
	25	0.4404	0.0015		0.4807
Rat-pooled fat	45	0.3847	0.0001		0.4481
	37	0.4037	0.0013		0.4588
	30	0.4219	0.0011		0.4755
	25	0.4363	0.0060		0.4762

[a] Parenthesis refers to temperature which differs from present study. [b] See reference 6.

TABLE III.—SOLUBILITIES OF XENON IN LIPIDS

Lipid	Temp., °C.	Bunsen's Absorption Coefficient—Mean	Bunsen's Absorption Coefficient—S.D.	Lit. Value[a]	Ostwald Solubility Coefficient
Olive oil	45	1.4839	0.0012		1.7248
	37	1.6307	0.0014	1.7[b]	1.8532
	30	1.7857	0.0013		1.9749
	25	1.8988	0.0014	1.9 (22°)[b] 1.8 (20°)[c]	2.0725
Dog fat	45	1.4589	0.0015		1.6962
	37	1.6113	0.0021		1.8299
	30	1.7557	0.0020		1.9493
	25	1.8393	0.0025		2.0084
Human fat 1	45	1.4753	0.0015		1.6904
	37	1.6143	0.0022		1.8345
	30	1.7688	0.0025		1.9630
	25	1.8878	0.0032		2.0606
Human fat 2	45	1.4742	0.0028		1.7171
	37	1.6251	0.0025		1.8503
	30	1.7645	0.0025		1.9583
	25	1.8476	0.0026		2.0166
Rat-pooled fat	45	1.4276	0.0045		1.6627
	37	1.5712	0.0050		1.7856
	30	1.7197	0.0055		1.9086
	25	1.8376	0.0065		2.0057

[a] Parentheses refer to temperatures which differ from present study. [b] See reference 6. [c] See reference 5.

where D_2 and D_1 are densities of oil or fats at temperatures t_2 and t_1, respectively. K is equal to 0.00066, a universal constant for all the oil and fats, except the rat-pooled fat, where K, equal to 0.0007 was used to fit the data obtained at 25 and 30°.

Measurement of the Viscosity and Surface Tension of Lipids.—The viscosities of olive oil and fats were determined using a Hoeppler precision viscometer. The surface tensions of the oil and fats were measured with a Cenco du Nouy Surface and Interfacial Tensiometer, model 70545, using a platinum-iridium ring of 5.996 cm. mean circumference. The ratio of the radius of the ring to that of the wire of the ring (R/r) is 53.9. Measurements were made in 100-ml. beakers, having an inside diameter of approximately 5 cm. All experimental values obtained were corrected by applying the Zuidema and Waters correction equation (12). The surface tensions of the oil and fats were also measured with a Traube stalagmometer and calculated using the surface tension of benzene and toluene at 20 and 30° as reference. Five to eight determinations of the viscosity and surface tension were made for each sample. The temperature was maintained by circulation of water from a thermostat water bath.

RESULTS AND DISCUSSION

The solubility of carbon dioxide, krypton, and xenon in olive oil, dog fat, rat-pooled fat, human fats No. 1 and No. 2 was measured at temperatures of 25, 30, 37, and 45°. Each sample was measured three times. The error of those measurements will be predominantly due to the barometric pressure reading, which is assumed to have ± 0.5 mm. error and the temperature setup, which is assumed to have ±0.05° flexibility. Therefore, for the large solubility of xenon in lipids, it will cause an approximate 0.2% deviation, and for the smaller solubility of carbon dioxide and krypton, it will cause an approximate 0.15 and 0.1% deviation, respectively.

The results obtained from these measurements, in terms of Bunsen's absorption coefficient, α, and of Ostwald's solubility coefficient, L, are presented in Tables I, II, and III. The relationship between L and α is $L = \alpha T/T_o$. The literature values taken were reduced in terms of Bunsen's absorption coefficient for uniform comparison. The data obtained from the present study agree well with the data of Lawrence, *et al.* (6), who measured the solubility of radioactive krypton and xenon in olive oil at 22 and 37°.

It is very interesting to note that although the fatty acid compositions of the oil and fats differ, the respective solubilities of carbon dioxide, krypton, and xenon are essentially the same in the oil as in the fats. Using olive oil as a standard, the deviation of the solubility ratio of the fat to olive oil is ±0.15%. These findings agree with the data of Nussbaum (13), who reported that the solubilities of the highly fat-soluble inert gas, radon, in fatty acid and synthetic triglycerides, increases with an increase in the number of carbon atoms per mole of fatty acid. This holds up to 7 carbon atoms per molecule. There then "appears to be a slight, and possibly insignificant, decline in solubility as the number of carbon atoms per molecule increase above eight."

Effect of Temperature on Solubility.—For an isothermal process, the effect of temperature on solubility of gas in liquid can be obtained as follows (14)

$$\text{Log } L = \frac{-\Delta H^\circ}{2.303RT} + \frac{\Delta S^\circ}{2.303R}$$

Graphically, log L is a linear function of $1/T$. Heat of solution, ΔH°, and entropy of solution, ΔS°, can be calculated from the slope and intercept of the straight line. The solubility of carbon dioxide, krypton, and xenon in the oil and fats in present studies was found to decrease as temperature increased. In this respect, our data agree with the data of Clever, *et al.* (15), covering the solubility of krypton and xenon in nonpolar hydrocarbon solvents and perfluoromethylcyclohexane. Interestingly, Clever, *et al.* (15*a*), and Lanning (16) reported

that the solubility values of helium, neon, and argon in both nonpolar hydrocarbon solvents and polar organic solvents increased as the temperature rose. In plotting logarithms of the solubility of carbon dioxide, krypton, and xenon in the oil and fats against the reciprocal absolute temperature, straight lines were obtained. The heats and entropies of solution, calculated according to the least square method, are essentially the same in the oil as in the fats. The heat and entropy of solution for krypton is -1185 ± 46 cal./mole, -5.6 ± 0.10 cal./mole; for carbon dioxide -2713 ± 30 cal./mole, -8.5 ± 0.10 cal./mole; and for xenon -2273 ± 80 cal./mole, -6.4 ± 0.2 cal./mole, respectively.

Solubilities and Surface Tension of Solvent.—Assuming the energy change on transferring a gas molecule from the gas phase to the solution is the sum of two energy changes: (*a*) the energy expanded to create a cavity of gas molecule radius, γ, in the solvent and (*b*), an interaction energy that arises when the gas molecule is placed in the cavity; Uhlig (17) derived the following equation

$$\ln L = 4\pi r^2\sigma/kT + E/kT$$

which predicts that a linear relation should be formed between the logarithm of the Ostwald coefficient, L, and the solvent's surface tension, σ, assuming the interaction energy, E, is a constant for the various solvents, and K is Boltzmann's constant. Both Uhlig and Clever, *et al.* (15), found this relationship to be justified. However, Gjaldbaek (18) found this relationship does not hold for water to which surfactant has been added. Our data indicate that gas solubility increases with increasing surface tension of lipids. Plotting logarithm of Bunsen's absorption coefficient against the surface tension of the oil and fats at different temperatures, straight lines were obtained. This result is in contrast to the inverse relationship between surface tension and gas solubility in a variety of hydrocarbon solvents at one temperature deduced by Uhlig (17).

Solubility and Viscosity of Solvent.—Winkler (19) proposed an equation relating solubility to viscosity. The equation is

$$\frac{\alpha_1 - \alpha_2}{\alpha_1} = \frac{Z_1 - Z_2}{Z_1}\frac{\sqrt[3]{M_g}}{K}$$

in which Z_1 and Z_2 are the viscosities at temperatures t_1 and t_2. M_g is the molecular weight of the gas. For five diatomic gases in water, K was found to be nearly equal to the cube root of 54, three times the molecular weight of water. Steinberg and Manowitz (5) reported that the less viscous silicone oil (1 centistoke) had a 63% higher xenon solubility than the more viscous silicone oil (10 centistoke). Using our data and applying the Winkler equation, the calculated (average of 30 data) K values are 8.13, 21.7, and 13.8 for carbon dioxide, krypton, and xenon, respectively. Our data suggest that gas solubility decreases with a decrease in the viscosity of the lipids. Plotting logarithm of Bunsen's absorption coefficient against logarithm of viscosity of the oil and fats at different temperatures, straight lines were obtained. Our data are in agreement with the statement of Thorpe and Rodger (20). They stated that, "Winkler's conclusion must be changed to: for same gas, the decrease in solubility (not percentage decrease) is proportional to the corresponding decrease in viscosity, and further, for any gas, the factor of proportionality is greater for a greater molecular weight, but no simple relation exists."

The linear relationship obtained from a plot of the logarithm of the solubility, against the surface tension, or logarithm of the viscosity of the lipid, appears to be a cross-relation plot. These relations can also be considered as a relation between solubility and temperature, since the surface tension and viscosity change as temperature varies.

Past efforts to elucidate the forces and factors which determine solubility have provided information on surface tension (17), viscosity (5,19), and the empty volume (15*b*) of the solvent as being related to gas solubility. Since our studies involved lipid mixtures, it was impossible to calculate Bondi's empty volume (15*b*). Our data of surface tension and viscosity measurements of solvents as well as those reported in the literature (15,17) were ob-

TABLE IV.—SURFACE TENSION AND VISCOSITY

Lipid	Temp., °C.	Surface Tension, Dynes/cm. Uncorrected Mean	Uncorrected S.D.	Corrected Mean	Corrected S.D.	Drop weight Mean	Drop weight S.D.	Lit. Values[a]	Viscosity, Centipoises Mean	S.D.	Lit. Values[a]
Olive oil	45	33.55	0.07	30.05	0.06	30.10	0.04	33.8 (50°)[b]	29.91	0.14	24 (50°)[e]
	37	34.10	0.08	30.56	0.07	30.76	0.11	33.6[c]; 34 (40°)[b]	39.08	0.10	46.68[f]
	30	34.65	0.05	31.07	0.04	31.03	0.10	34.9[b]; 33.9 (32°)[c]	51.05	0.26	41 (33.5°)[c]
	25	35.02	0.04	31.41	0.03	31.47	0.25	34.2 (27°)[c]	61.90	0.13	77 (20°)[e]
Dog fat	45	25.22	0.10	22.20	0.09	22.35	0.17	34.2—35.4(20°)[d]	33.73	0.13	
	37	25.65	0.07	22.70	0.06	22.66	0.27		44.58	0.22	
	30	26.22	0.09	23.25	0.08	23.50	0.18		57.84	0.10	
	25	26.60	0.10	23.56	0.09	26.96	0.24		71.28	0.10	
Human fat 1	45	24.30	0.10	21.46	0.09	21.30	0.24		32.95	0.10	
	37	25.05	0.10	22.10	0.09	21.72	0.21		42.31	0.09	
	30	25.54	0.10	22.59	0.09	22.30	0.25		55.68	0.17	
	25	26.96	0.11	22.96	0.10	22.63	0.29		68.29	0.09	
Human fat 2	45	23.52	0.04	20.75	0.04	20.57	0.20		32.37	0.10	
	37	24.10	0.10	21.25	0.09	21.16	0.24		48.46	0.13	
	30	24.54	0.11	21.69	0.10	21.51	0.26		56.98	0.05	
	25	24.92	0.11	22.05	0.10	...	...		69.43	0.15	
Rat-pooled fat	45	24.71	0.10	21.98	0.09	22.24	0.08		33.94	0.07	
	37	25.85	0.14	22.85	0.13	23.43	0.16		44.77	0.09	
	30	26.70	0.06	23.66	0.05	23.80	0.28		59.21	0.08	
	25	27.32	0.04	24.22	0.03	24.31	0.10		72.81	0.05	

[a] Parentheses refer to temperatures which differ from present study. [b] See reference 24. [c] See reference 26. [d] See reference 25. [e] See reference 27. [f] See reference 28.

tained with the solvents saturated with air. No data are available for measurements with degassed solvents or solvents saturated with the experimental gas.

The vapor pressures of the oil and fats, which are involved in the calculation of the solubilities, are negligible. The vapor pressures of olive oil and hydrogenated cottonseed oil have been reported as 0.001, 0.37, 0.01, and 0.04 mm. at 250°, respectively (21–23). Likewise, the natural fats have very low pressures.

The uncorrected surface tensions (maximum pull P) of the olive oil in the present study agree well with the data of Halpern (24), of Kaufmann and Kirsch (25), and of Canals, *et al.* (26). After applying the Zuidema and Waters correction (12), the surface tensions of the oil and fats of our present study were 10% lower than the data of Halpern, of Kaufmann and Kirsch, and of Canals, *et al.*, which we assumed are uncorrected. To further check this point, the drop weight method has been used to measure the surface tension of oils and fats over the same range of temperatures. For the purposes of comparison, uncorrected and corrected surface tensions of the lipids are presented in Table IV.

SUMMARY

1. The solubility of carbon dioxide, krypton, and xenon in olive oil, dog fat, rat-pooled fat, and human fats, has been measured at one atmosphere and at temperatures of 25, 30, 37, and 45°. The solubility of these gases in the above mentioned oil and fats is essentially the same, except for the rat-pooled fat, which is slightly less.

2. The heats and entropies of solution, calculated according to the least square method, are essentially the same in the oil as in the fats.

3. The viscosity and surface tensions of the oil and fats have also been measured at the temperatures of 25, 30, 37, and 45°. The density of olive oil and fats has been measured at the temperatures of 25 and 30°.

TABLE V.—DENSITY OF LIPIDS

Lipid	Temp., °C.	Density $t/4°$ Mean	Density $t/4°$ Lit. Value
Olive oil	45	0.9011	
	37	0.9064	
	30	0.9110	
	25	0.9143	
	15	0.9150	0.915–0.920[a]
Dog fat	45	0.8974	
	37	0.9027	0.9155[b]; 0.9103[c]
	30	0.9073	
	25	0.9116	0.9118[c]
Human fat 1	45	0.8965	
	37	0.9017	0.9110[b]; 0.9000[c]
	30	0.9064	
	25	0.9097	0.9090[c]
	15	0.9163	0.9167[c]
Human fat 2	45	0.8972	
	37	0.9025	
	30	0.9072	
	25	0.9104	
Rat-pooled fat	45	0.9002	
	37	0.9058	0.9154[b]; 0.9038[c]
	30	0.9107	
	25	0.9142	0.9121[c]

[a] See reference 11*a*. [b] See reference 4. [c] See reference 29.

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Pithecolobium dulce I

Isolation and Characterization of Legume Constituents

By S. K. NIGAM, R. K. GUPTA†, and C. R. MITRA

The seed and the mesocarp of the *P. dulce* legumes were worked up separately. The alcoholic extract of the seed powder yielded (I) a saponin, m.p. 175–181°; sapogenin provisionally named pithogenin, $C_{28}H_{44}O_4$, m.p. 207–208°; $[\alpha]_D^{17°}$ + 81°; elemental analyses and the spectrophotometric data suggest it to be a steroid genin; (II) a sterol-glucoside-B, m.p. 276–278°; the aglycone, m.p. 159–160°; and (III) a flavone, m.p. 298–306°. The seed fat (20%) similar to the leguminous fats in general, was purified and refined and the characteristics noted. The seed also yielded pure lecithin (0.7%). The sweet pulpy mesocarp yielded hexacosanol and a sterol-glucoside-A, m.p. 282–286°; aglycone, m.p. 136–138°. The sugar (66.5%; mostly glucose) and the amino acids, L-proline, L-leucine, L-valine, and asparagine were present.

PITHECOLOBIUM DULCE, Benth., N. O. *Leguminosae* (habitat: Mexico and Deccan peninsula of India), is extensively cultivated as a hedge plant. The shrub is hardy and stands frequent pollarding. It is reported in folk medicine to be useful in the treatment of leprosy (1). A few species of this genus are toxic. Alkaloidal principles have been isolated from *P. saman* (2). The bark and beans of another species, *P. lobatum* (3), are reported to cause dysuria and colic. As no work appears to have been carried out with the *P. dulce* legume, systematic chemical examination of the legumes was undertaken.

The ripe legumes consist of six to eight hard, black, flattened seeds embedded in a sweet pulpy mesocarp. The mesocarp appears to be edible, but it is reported to cause colic and gives a tickling sensation to mouth mucosa.

Constituents of Mesocarp

The residue from the alcohol-miscible free-flowing syrup, obtained from the mesocarp on extraction with nonpolar solvents, yielded a waxy fraction and a sterol-glucoside-A, m.p. 282–286° in yields of 0.8% and 0.02%, respectively. The fatty alcohol constituting the major portion of the waxy fraction was identified as hexacosanol. The sterol-glucoside-A yielded on hydrolysis an apparently new sterol-aglycone-A, m.p. 136–138°; the sugar moiety of the glucoside has been identified as glucose through paper co-chromatography in two solvent systems (*n*-butanol 4, acetic acid 1, water 5; and pyridine 1, ethyl acetate 2, water 2; aniline phthalate spray). The dextrorotation and other characteristics of the sterol-aglycone-A (Table I) differ from those of the sito-sterols and so also of the parent sterol glucoside-A from the β-D-glucoside of β-sitosterol (4).

The alcohol-soluble fraction of the mesocarp, when examined in aliquot portions, showed the presence of 66.5% of total sugar of which reducing sugar [estimated as glucose; iodometric (5)] was found to be 62.9% of the alcohol-soluble total solid. The paper chromatograms in two solvent systems (same as above) showed the presence of two spots of glucose and fructose but no sucrose was indicated. The alcoholic extract of the mesocarp yielded an osazone identical with authentic glucosazone. The amino acids, L-proline, L-leucine, L-valine, and asparagine were identified by paper chromatography (*n*-butanol 4, acetic acid 1, water 5; ninhydrin spray) while an additional spot in the chromatogram remained unidentified (6). The more soluble (aqueous) fraction of the alcoholic extractive showed the presence of traces of a saponin and a flavone.

Constituents of the Seed

In addition to 20% fixed oil, an appreciable quantity of a saponin (*ca.* 2.4%) as well as lecithin (*ca.* 0.7%) and a flavone (*ca.* 0.0001%) have been isolated from the seed for the first time. Generally, leguminous plants do not bear saponins, the subfamily, *Papilionoideae* (7) and the genera, *Albizzia* (8) and *Acacia* (9) being a few exceptions. An unidentified saponin has so far been indicated to be present in only one species of this genus, *P. cauliflorum* (10).

In order to isolate the different constituents of the seed under mild conditions and to avoid interference of the fat towards the isolation of the lipid-associates, such as phosphatides, powdered seed material was at first exhaustively percolated with alcohol and the fat in the residual meal was subsequently extracted with hydrocarbon solvent. The extractives from both the solvents were worked up separately. Concentration of the alcoholic extract deposited microcrystalline needles of sterol-glucoside-B, m.p. 276–278° (*ca.* 0.007%) which had glucose as the sugar component (confirmed through paper chromatography). The sterol-aglycone-B melted at 159–160°.

The mother liquor, freed of alcohol under reduced pressure, was macerated successively with excess of

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TABLE I.—CHARACTERISTICS OF THE STEROLS OF *P. dulce* LEGUME

	Sterol-aglycone-A	Sterol-aglycone-B	Sterol from Phosphatidic Fraction
Melting point	136–138°	159–160°	159–160°
Optical rotation	+37°	−44°	...
I.R. (Nujol; cm.$^{-1}$)	3279, 2817, 2532, 1695, 1450, 1366, 1163, 1129, 1082, 1048, 966,	3380, 1713, 1641, 1600, 1323, 1254, 1216, 1197, 1155, 1141, 1103, 1067, 1047, 1040, 1018, 971, 939, 892, 869, 846, 832.	...
Acetate: m.p.	119°	176°	176°
$[\alpha]_D$	−16°	−16.4°	−16.0°
Benzoate: m.p.	192°	185°	185°

petrol-ether (b.p. 40–60°), ether, and ethyl acetate. Four well-defined fractions were obtained: (*a*) petrol-ether soluble, (*b*) petrol-ether insoluble and ether soluble, (*c*) ethyl acetate soluble but petrol-ether and ether insoluble and (*d*) alcohol-water soluble but insoluble in petrol-ether and ether. The dark green semisolid petrol-ether soluble fraction (*a*) on digestion with acetone yielded acetone-insoluble phosphatidic material, and the acetone solution yielded the glycerides. The phosphatidic fraction on usual purification and fractionation with alcohol yielded lecithin, $[\alpha]_D^{28°}$ + 14° (c, 1.0; $CHCl_3$); nitrogen, 1.0% (11), as the major component. The sterol, isolated from the unsaponifiable fraction of the phospholipids of the seed, was identical with the sterol-aglycone-B (Table I; the mixed melting points of both the sterols as well as of their acetates and the benzoates were undepressed). Obviously, the phytosterol present in the seed is different from that of the mesocarp. Further studies in the sterols are being continued.

The ether- and ethyl acetate-soluble fractions (*b*) and (*c*) yielded a very small quantity of a flavone melting at 298–306°; $\lambda_{max.}^{EtOH}$ 270, 349 mμ, $\lambda_{min.}^{EtOH}$ 248 mμ; color tests: magnesium hydrochloric acid, orange-red; ferric chloride, dark olive-green; and lead acetate, bright lemon-yellow; its acetate melted at 208–209°. The paper chromatogram (*n*-butanol 5, acetic acid 1, water 4) of the flavone confirmed it to be a single substance.

The dilute alcohol-soluble fraction (*d*) of the seed extractive manifested considerable frothing on removal of most of the solvent under reduced pressure and on usual working. This fraction yielded a saponin as a white foam, m.p. 175–181°; $[\alpha]_D^{25°}$ + 48.5° (c, 1.0; 60% alcohol). Graded hydrolysis with 2–6% hydrochloric acid of the saponin gave the genin which, after purification through and regeneration from its acetate, melted at 207–208°; $[\alpha]_D^{17°}$ + 81° (c, 1.0; $CHCl_3$). Persistent difficulty was encountered in isolating the genin in workable yield by conventional hydrolysis of the saponin. Mild hydrolysis even at stages did not give satisfactory results. The crude genin was mostly resinous and repeated attempts for purification through chromatography using different eluents met with little success. Crude genin acetate was, therefore, prepared under mild conditions and purified through chromatography. In either case, the ultimate yield of the genin was very poor. However, the purity of

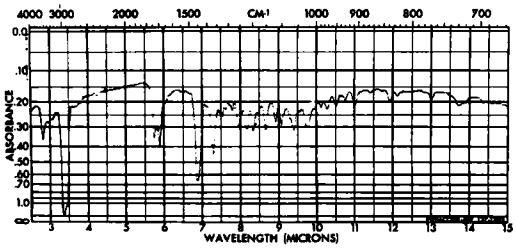

Fig. 1.—Spectrophotometric data of pithogenin.

the genin obtained through the acetate was ensured by repeated chromatography. A tentative molecular formula, $C_{28}H_{44}O_4$, was arrived at through elemental analyses of the genin and its acetate. The genin, provisionally named pithogenin, is LB-positive (pink to violet); with thionyl chloride-ferric chloride it develops orange to red; with thionyl chloride and antimony trichloride, a yellow to orange color (12). Spectrophotometric data of pithogenin [infrared bands at 3569, 2959, 2882, 1742, 1701, 1453, 1370, 1009, 988, 973, 951, 928, 907, 854, 839, cm^{-1} (Fig. 1); ultraviolet absorption (Fig. 2), $\lambda_{inflex.}^{EtOH}$ 260 mμ (ε, 772.4)] indicate the presence of hydroxyl and an isolated carbonyl group which suggest a similarity with steroid sapogenins (13, 14); further correlating data are being collected. The sugar moiety of the saponin was found to be gluclose identified through paper co-chromatography and confirmed through the mixed melting point of its osazone with glucosazone.

The seed fat obtained as a greenish oil was processed free from alcohol-soluble material, refined and bleached (15), and showed the following characteristics: color (Lovibonds, 1-cm. cell), 1.1 Y; sp. gr. (26°), 0.9064; $n_D^{26°}$, 1.4635; acid No., 0.2; sap. No., 183; iodine value (Wij's), 68; and unsaponifiable, 1.07%.

EXPERIMENTAL[1]

Examination of Mesocarp

Mature legumes used in this investigation were collected during May and June. Average weight of a legume was 25 Gm. and that of a seed, 110 mg.

[1] Melting points are uncorrected.

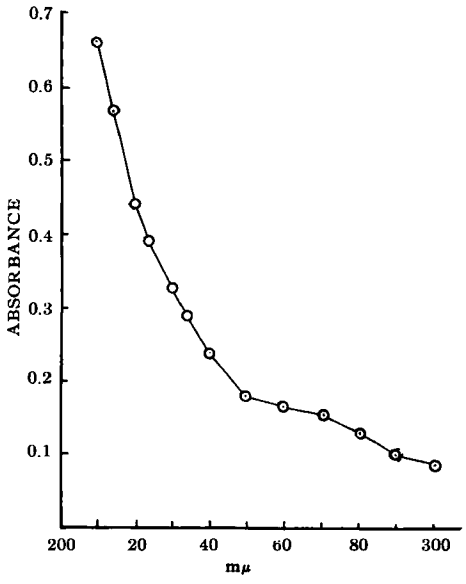

Fig. 2.—Ultraviolet absorption of pithogenin: $\lambda_{\text{inflex.}}^{\text{EtOH}}$ 260 mμ (ε, 772.4).

The seeds were separated from the pulpy mesocarp freed of brown brittle legume coat.

Sterol-glucoside-A.—Seventeen kilograms of air-dried sticky mesocarp was macerated and percolated with 21 L. alcohol five times until the alcohol insoluble material (*ca.* 10% of the air-dried mesocarp) was left behind mostly as fibrous residue. On removal of major bulk of alcohol by distillation under reduced pressure, a dark brown, syrupy, free-flowing mass was obtained which was successively extracted with 4 L. petrol-ether (b.p. 40–60°) three times, 4 L. ether three times, and 5 L. ethyl acetate twice. During this processing 3.1 Gm. sterol-glucoside-A separated out as microcrystalline shiny needles. It was further purified by crystallization from 2 L. of 90% alcohol and the final product melted at 282–286°, $[\alpha]_D^{25°}$ −20° (c, 1.0; pyridine).

Anal.—Calcd. for $C_{35}H_{60}O_6$: C, 72.9; H, 10.42. Found: C, 71.7; H, 10.7.

Sterol-aglycone-A.—Hydrolysis of 230 mg. of sterol-glucoside-A was effected by refluxing it with 75 ml. alcoholic hydrochloric acid (5% v/v) for 5 hours. After cooling the reaction mixture, the precipitated aglycone was taken up in ether and worked up. Its ether-petrol-ether mixture (90:10) was passed through a column of 5 Gm. alumina, and the resultant product on crystallization from alcohol-ether (90:10) yielded the sterol, m.p. 136–138°; $[\alpha]_D^{17°}$ + 37° (c, 1.0; $CHCl_3$).

Anal.—Calcd. for $C_{29}H_{50}O$: C, 84.07; H, 12.1. Found: C, 83.8; H, 12.2.

Sterol-aglycone-A-acetate.—The sterol aglycone-A (100 mg.) was dissolved in 1 ml. pyridine and refluxed with 2 ml. acetic anhydride for 8 hours at 110° and the reaction mixture worked up in the usual manner. The acetate was purified by passing its ether-petrol-ether (80:20) solution through a column of 5 Gm. alumina. When finally crystallized from alcohol it melted at 118–119°; $[\alpha]_D^{17°}$ − 16° (c, 1.0; $CHCl_3$).

Anal.—Calcd. for $C_{31}H_{52}O_2$: C, 81.53; H, 11.4. Found: C, 81.45; H, 11.2.

Sterol-aglycone-A-benzoate.—Fifty milligrams of the aglycone was taken up in 1 ml. pyridine and 2 ml. benzoyl chloride added dropwise. The reaction mixture was left at room temperature (20–35°) for 24 hours. The benzoate, on usual working and purification by passing its ethereal solution through a column of 4 Gm. alumina and on subsequent crystallization from alcohol, melted at 192°.

Anal.—Calcd. for $C_{36}H_{54}O_2$: C, 83.4; H, 10.4. Found: C, 83.1; H, 10.4.

Hexacosanol.—The ether, petrol-ether, and ethyl acetate soluble fractions, separately worked up, yielded 13.2 Gm. of waxy compound having a melting range of 65–75°. On repeated crystallizations from petrol-ether, hot alcohol, and alumina column chromatography using petrol-ether as eluent, the waxy compound yielded hexacosanol as a white microcrystalline product, m.p. 79°, mixed melting point with authentic sample 78–79°.

Anal.—Calcd. for $C_{26}H_{54}O$: C, 81.7; H, 14.1. Found: C, 81.5; H, 14.2.

Hexacosanol Acetate.—This was prepared with acetic anhydride and sodium acetate and after usual working and crystallization from alcohol it melted at 64°.

Anal.—Calcd. for $C_{28}H_{56}O_2$: C, 79.2; H, 13.2. Found: C, 79.3; H, 13.4.

Examination of the Seed

Powdered seeds weighing 8.8 Kg. were percolated at room temperature (15–30°) successively with 7 L. 95% alcohol four times and 7 L. petrol-ether (b.p. 60–80°) three times.

Sterol-glucoside-B.—The alcoholic percolate on concentration under reduced pressure below 50° deposited 0.8 Gm. microcrystalline sterol-glucoside-B which when recrystallized, as in the case of sterol-glucoside-A, melted at 276–278°; $[\alpha]_D^{25°}$ −25° (c, 1.0; pyridine).

Anal.—Calcd. for $C_{34}H_{58}O_6$: C, 72.6; H, 10.3. Found: C, 71.9; H, 10.2.

Sterol-aglycone-B.—Hydrolysis of 200 mg. of sterol-glucoside-B was carried out as in the case of sterol-glucoside-A to yield the sterol-aglycone-B which on usual purification and crystallization from alcohol melted at 159–160°; $[\alpha]_D^{36°}$ −44° (c, 1.0; $CHCl_3$).

Anal.—Calcd. for $C_{28}H_{48}O$: C, 84.0; H, 12.0. Found: C, 82.8; H, 12.0.

Sterol-aglycone-B-acetate.—Prepared in the usual way, the acetate on crystallization from alcohol melted at 176°; $[\alpha]_D^{34°}$ −16.4° (c, 1.0; $CHCl_3$).

Anal.—Calcd. for $C_{30}H_{50}O_2$: C, 81.4; H, 11.3. Found: C, 80.3; H, 10.6.

Sterol-aglycone-B-benzoate.—This was also prepared by heating the substance with benzoyl chloride in pyridine at 110° for 3 hours. The benzoate after repeated crystallizations from alcohol melted at 185°.

Anal.—Calcd. for $C_{35}H_{52}O_2$: C, 83.3; H, 10.3. Found: C, 82.7; H, 10.0.

The mother liquor after removal of the sterol-glucoside-B was further concentrated under reduced pressure when the resultant aqueous solution manifested copious frothing. It was ice cooled and extracted successively with 4 L. petrol-ether (b.p. 40–60°) three times, 4 L. ether three times, and 3 L.

ethyl acetate three times when finally a gelatinous mass was left in the aqueous phase. The petrol-ether soluble fraction obtained on removal of solvent finally *in vacuo* yielded 200 Gm. greenish residue which when repeatedly agitated with acetone, finally gave 54 Gm. acetone-insoluble phosphatidic material. The phospholipoid fraction was further extracted with cold alcohol and the residue, after removal of alcohol under reduced pressure, was purified by acetone and ether to yield 27.5 Gm. cream-white lecithin. The alcohol-insoluble phosphatidic material was then extracted with hot alcohol to yield a further quantity of 21.6 Gm. of alcohol soluble lecithin and 4.9 Gm. of alcohol-insoluble cephalin. Twenty-one grams of lecithin fraction on acid hydrolysis and subsequent saponification gave 0.8 Gm. of sterol, m.p. 160°. The acetate and the benzoate of the sterol were prepared as in the foregoing cases for comparison (Table I).

The ether and ethyl acetate soluble fractions yielded only the flavone.

Saponin and Pithogenin.—The gelatinous precipitate from the aqueous fraction of the seed extractive was dissolved in 300 ml. alcohol and 3 L. ether slowly added; the saponin precipitated out. On repeating the process with aliquot portions several times, 211 Gm. of the saponin was finally obtained as white foam, m.p. 175–181°, on removal of last traces of solvent *in vacuo*.

Saponin Acetate.—To a solution of 600 mg. of the saponin in 5 ml. pyridine, 25 ml. of acetic anhydride was added and the mixture left for 48 hours at room temperature (20–25°). The reaction mixture was next macerated with crushed ice and the precipitated saponin acetate was washed with water until free from acid. It was crystallized from alcohol and melted at 138°.

Pithogenin.—Ninety-four grams of saponin was hydrolyzed in 300 ml. of 90% alcohol with 2% hydrochloric acid at 20–35° for 24 hours. The cooled reaction mixture was extracted with 400 ml. of benzene. After separation of the benzene extract, the aqueous fraction was heated to 90° for 24 hours, cooled and extracted with benzene. The process was repeated five times with successive 1% increments in the acid percentage up to 6%. The combined benzene extract on usual working yielded 4.8 Gm. of crude genin as a dark brown product. Alumina column chromatography of 2.4 Gm. of genin using benzene-chloroform mixture (80:20) as the eluent yielded only 6 mg. of crystalline genin, melting at 195°, softening earlier.

Pithogenin Acetate.—This was prepared by heating 2.4 Gm. of the crude genin with 30 ml. of acetic anhydride and 8 Gm. of freshly fused sodium acetate on a steam bath for 72 hours. The reaction product was worked up in the usual manner. The genin acetate was eluted in a column of 20 Gm. alumina with ether-petrol-ether mixture (75:25) and six fractions were collected. The fourth and fifth fractions gave 120 mg. crystalline acetate which was further crystallized from ether-petrol-ether mixture (50:50) to yield a colorless acetate, m.p. 176–180°.

Anal.—Calcd. for $C_{28}H_{43}O_3 \cdot OCOCH_3$: C, 74.1; H, 9.5. Found: C, 73.8; H, 9.8.

Regeneration of Pithogenin.—Pithogenin was regenerated from 100 mg. of acetate with 10 ml. of 5% sodium carbonate solution at ordinary temperature. After usual working, 70 mg. of genin was obtained and purified by chromatography using ether-petrol-ether (85:15) as the eluent. Fifty milligrams of the product thus obtained were crystallized from ether-alcohol (90:10) to yield silky-white needles of pithogenin, m.p. 207–208°, $[\alpha]_D^{17°}$ +81° (c, 1.0; $CHCl_3$).

Anal.—Calcd. for $C_{28}H_{43}O_3OH$: C, 75.6; H, 9.6. Found: C, 75.3; H, 10.1.

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Effect of Chlorination on the Anticonvulsant Activity of Phenobarbital

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The lethal doses, neurotoxic doses, anticonvulsant potencies, and protective indices for two chlorinated barbiturates, 5-(4-chlorophenyl)-5-ethylbarbituric acid (monochloro) and 5-(3,4-dichlorophenyl)-5-ethylbarbituric acid (dichloro), were determined in mice and the results compared with similar values concomitantly determined for phenobarbital. Chlorination was found to delay markedly the time for peak anticonvulsant effect and to prolong duration of action. The lethal dose for monochloro was somewhat larger and that for dichloro significantly smaller than that for phenobarbital. The minimal neurotoxic dose for monochloro was significantly larger than that for either dichloro or phenobarbital. All three compounds exhibited anticonvulsant activity in nontoxic doses. Although chlorination of phenobarbital had no significant effect on protective indices, the protective indices for dichloro tended to be somewhat higher than those for the other two compounds.

NUMEROUS compounds have been halogenated in an effort to improve their pharmacological properties and/or margins of safety. To cite a few examples, some anti-inflammatory steroids (1), thiazide diuretics (2), antihistaminics (3), and phenothiazine ataractics (4) have been subjected to such chemical manipulation. Therefore, the availability of two chlorinated analogs of phenobarbital provided an opportunity to study the effects of chlorination on the anticonvulsant activity of an agent with well-established clinical usefulness.

METHODS

Male albino mice (Carworth Farms, CF No. 1 strain) were used as experimental animals. They were maintained on Rockland mouse diet and allowed free access to food and water except for the short time they were removed from their cages for testing. The following anticonvulsant agents were studied: 5-(4-chlorophenyl)-5-ethylbarbituric acid (monochloro), 5-(3,4-dichlorophenyl)-5-ethylbarbituric acid (dichloro), and 5,5-phenylethylbarbituric acid (phenobarbital). All three agents were administered orally in 0.9% sodium chloride solution; but, in order to increase the solubility of the two chlorinated compounds, two drops of a 10% solution of sodium hydroxide were added to each 50 ml. of solvent.

Two tests were utilized to determine anticonvulsant potencies (ED_{50}'s): one electrical and one chemical. The test based on electrically induced convulsions measured the ability of the drug to prevent the hindleg tonic-extensor component of maximal electroshock seizures evoked by supramaximal current (MES test; 50 ma. alternating current, 0.2-sec. stimulus duration, corneal electrodes). The test based on chemically induced convulsions measured the ability of a drug to afford complete protection against seizures induced by the subcutaneous injection of pentylenetetrazol (Metrazol; 85 mg./Kg.; s.c. Met. test). The details of the techniques, the end points employed in mice, and the characteristics of the electroshock apparatus have been published elsewhere (5, 6). The dose lethal to 50% of animals within 24 hours after drug administration (LD_{50}) and the mean neurotoxic dose (TD_{50}) were also determined for each drug. The end point used to estimate minimal neurotoxicity was muscular incoordination, based on the inability of the animal to remain for one minute on a horizontal rod rotating at 6 r.p.m. Each drug was tested at the time of peak activity as measured by the maximal electroschock seizure (MES) test. For the determination of the ED_{50}, TD_{50}, or LD_{50}, groups of 8 to 12 mice were given various doses of drug until at least three points were established in the range between 0 and 100% seizure protection, minimal neurotoxicity, or lethality, respectively. The results obtained were plotted on logarithmic probability paper and a regression line was fitted to the plotted points by eye. From this plot of the data the respective ED_{50}, TD_{50}, LD_{50}, 95% fiducial limits, and protective index (P.I. = TD_{50}/ED_{50}) were calculated by the method of Litchfield and Wilcoxon (7).

RESULTS

The time courses of anticonvulsant activity, as measured by the MES test, are shown in Fig. 1.

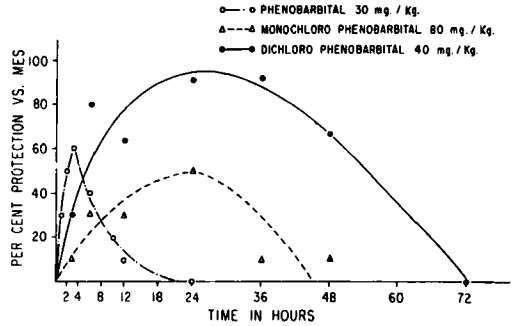

Fig. 1.—The anticonvulsant activity for phenobarbital and its monochloro and dichloro analogs.

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TABLE I.—EFFECT OF CHLORINATION ON THE NEUROTOXICITY AND ANTICONVULSANT PROPERTIES OF PHENOBARBITAL

Drug	Toxicity		Anticonvulsant Activity			
			MES Test		s.c. Met. Test	
	LD_{50}[a] mg./Kg.	TD_{50}[b] mg./Kg.	ED_{50} mg./Kg.	P.I.	ED_{50} mg./Kg.	P.I.
Phenobarbital	250 (219–285)	70 (59–83)	27.5 (22.2–34.1)	2.55 (1.92–3.35)	24.5 (19.3–31.1)	2.86 (2.12–3.86)
Monochloro	290 (259–325)	215 (169–273)	125 (106–148)	1.72 (1.27–2.32)	60 (48–75)	3.58 (2.59–4.94)
Dichloro	190 (158–220)	84 (74–96)	26.5 (17.4–40.2)	3.16 (2.06–4.88)	18.4 (12.5–27.1)	4.57 (3.04–6.84)

[a] Twenty-four-hour observation period. [b] Minimal neurological toxicity. Values in parentheses are 95% fiducial limits.

As indicated in this figure, chlorination markedly altered the time of peak anticonvulsant activity and the duration of action. Phenobarbital exerted its peak anticonvulsant effect 3 hours after oral administration, whereas the two chlorinated analogs exhibited maximum activity 24 hours after such treatment. Thus, chlorination extended the time of peak response approximately eightfold. Chlorination of phenobarbital also prolonged the duration of anticonvulsant activity. As may be calculated from the figure, it required 5 hours for the peak anticonvulsant activity of phenobarbital to fall 50%, whereas it required 14 and 30 hours for the activity of monochloro and dichloro, respectively, to decline to a similar degree.

The LD_{50}'s, TD_{50}'s, anticonvulsant ED_{50}'s, and protective indices for the chlorinated compounds, in comparison with those for phenobarbital, are shown in Table I. Except for the LD_{50}'s, which were determined 24 hours after drug administration, all tests were conducted at the time of peak drug activity as derived from the figure (3 hours for phenobarbital and 24 hours for the two chlorinated compounds). Chlorination had no dramatic effect on the LD_{50} for phenobarbital. The LD_{50} for monochloro was somewhat higher than that for phenobarbital, whereas the LD_{50} for dichloro was on the borderline of being significantly lower. Dichloro and phenobarbital induced overt symptoms of neurotoxicity in 50% of the animals after doses of 84 and 70 mg./Kg., respectively, whereas the TD_{50} for monochloro was significantly larger (215 mg./Kg.) than that for phenobarbital.

With regard to anticonvulsant potencies, the data in Table I indicate that monochloro was considerably less potent than either phenobarbital or dichloro by the two tests utilized. For example, the ED_{50} and 95% fiducial limits by the MES test were 27.5 (22.2–34.1) mg./Kg. for phenobarbital and 125 (106–148) mg./Kg. for monochloro. However, there was no significant difference between the ED_{50} for phenobarbital and that for dichloro by either the MES test or the s.c. Met. test.

When compared on the basis of protective indices (P.I.'s), it may be seen that the two chlorinated compounds do not differ significantly from phenobarbital. The P.I.'s by the MES test were 1.72 and 3.16 for monochloro and dichloro, respectively, as compared to 2.55 for phenobarbital. On the other hand, the P.I.'s as determined from data obtained by the s.c. Met. test were 2.86, 3.58, and 4.57 for phenobarbital, monochloro, and dichloro, respectively. The P.I.'s of all three compounds were higher by the s.c. Met. test than by the MES test, but the difference was significant only for monochloro.

DISCUSSION

The data presented indicate that chlorination of phenobarbital extends the time required for the drug to exert peak anticonvulsant effect, prolongs the duration of anticonvulsant action, alters the ratio of the LD_{50} to the TD_{50}, and may modify anticonvulsant activity. These observations are in agreement with the findings of Gibson and coworkers (8) in rats. The time required to reach peak anticonvulsant activity was approximately 8 times longer for monochloro and dichloro than for phenobarbital. The time required for peak anticonvulsant activity to fall 50% was found to be 3 and 7 times longer for monochloro and dichloro, respectively, than for phenobarbital.

It is interesting to note that the dose fatal to 50% of mice within a 24-hour time period is not inextricably related to the dose which causes minimal neurological deficit in a similar percentage of animals. For example, the TD_{50} for monochloro was significantly higher than that for phenobarbital, whereas the LD_{50}'s for the two compounds were not significantly different. On the other hand, the LD_{50} for dichloro was significantly lower than that for phenobarbital, whereas the TD_{50}'s for the two compounds were not significantly different. Thus, the ratio of the LD_{50} to the TD_{50} is 3.6, 1.3, and 2.3 for phenobarbital, monochloro, and dichloro, respectively. This indicates that chlorination modifies the lethal dose and the minimal neurotoxic dose independently.

With respect to anticonvulsant activity, monochloro is significantly less potent than either phenobarbital or dichloro. There is no significant difference in the anticonvulsant activity of phenobarbital and dichloro as estimated by the two tests employed.

An evaluation of the P.I.'s derived from the experimental data indicates that all the drugs have a higher margin of safety by the s.c. Met. test than by the MES test, but this difference is significant only in the case of monochloro. The P.I. for monochloro is less than that for phenobarbital by the MES test and more than that for phenobarbital by the s.c. Met. test. On the other hand, the P.I.'s for dichloro tend to be higher by both tests than those for phenobarbital, but the differences are not statistically significant.

A study of structure-activity relations suggests that 4-phenyl chlorination of phenobarbital decreases minimal neurotoxicity and anticonvulsant potency, whereas 3,4-dichlorophenyl substitution has no significant effect on either minimal neurotoxicity or anticonvulsant potency. The chlorination of phenobarbital markedly prolongs the dura-

tion of anticonvulsant activity. The more favorable anticonvulsant effects were observed when the phenyl ring of phenobarbital was chlorinated in the 3,4 positions.

Preliminary clinical trial (9) suggested that dichloro in a dose of 300 mg. per day has no significant anticonvulsant activity in man. This was unexpected since the results presented herein for mice and those reported by Gibson, *et al.* (8), for rats indicate that, except for its temporal properties, dichloro has a profile of anticonvulsant action remarkably similar to that of phenobarbital. In view of this dichotomy and because of the well-known usefulness of phenobarbital in epilepsy, the clinical value of dichloro should be unequivocally determined in order to test the validity of current laboratory procedures for screening potentially useful antiepileptic drugs.

SUMMARY

The anticonvulsant potencies (ED_{50}'s) of two experimental chlorinated barbiturates, 5-(4-chlorophenyl)-5-ethylbarbituric acid (monochloro) and 5-(3,4-dichlorophenyl)-5-ethylbarbituric acid (dichloro), and for phenobarbital were determined in mice by the following two tests: maximal electroshock seizure pattern (MES) test and pentylenetetrazol (Metrazol) seizure threshold (s.c. Met.) test. In addition, the dose of each drug fatal to 50% of animals (LD_{50}) and the dose which induced minimal evidence of neurotoxicity in 50% of animals (TD_{50}) were determined. Protective indices (P.I. = TD_{50}/ED_{50}) were calculated. On the basis of the results obtained the following conclusions appear to be justified.

1. Chlorination of phenobarbital modifies the lethal dose independently of the minimal neurotoxic dose.

2. Chlorination extends approximately eightfold the time required for the drug to exhibit peak anticonvulsant activity and prolongs three- to sevenfold the time for anticonvulsant activity to fall 50%.

3. The chlorinated barbiturates exhibit anticonvulsant activity by both tests; *monochloro* is less potent than phenobarbital, whereas the anticonvulsant potency of dichloro is not significantly different from that of phenobarbital.

4. On the basis of P.I.'s, dichloro exhibits the most favorable indices, but the P.I.'s for dichloro are not significantly different from those for phenobarbital. The P.I.'s for all three compounds are higher by the s.c. Met. test than by the MES test, but this difference is significant only in the case of monochloro.

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Structural Studies on the Triterpene Obliquol

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Oxidation studies have indicated that the two hydroxyl groups of obliquol are secondary, one of which exhibits some hindrance as in the Oppenauer oxidation. Nuclear magnetic resonance studies have supported the chemical evidence that the second hydroxyl group is in the 12 position in obliquol. The C-18 methyl peak of obliquol diacetate has shifted downfield from the corresponding peak in lanosterol acetate due to perturbation by the 12-acetyl group. Therefore, it was concluded that obliquol has the structure I. The complete NMR spectra of obliquol, lanosterol, and their acetates are interpreted.

IN A PREVIOUS communication (1) there was reported the isolation of a new triterpene, obliquol (I), from the fungus, *Poria obliqua* (Bres.). At that time, obliquol (I) was shown to be a tetracyclic triterpene with two hydroxyl groups. It was shown to possess two double bonds, one of which was shown to be unreactive and assigned to the 8,9 position. The possibility, based on infrared data, was proposed that one of the hydroxyl groups was primary. This has since been found to be a secondary hydroxyl group. Additional studies on the structure of obliquol comprise the contents of this communication.

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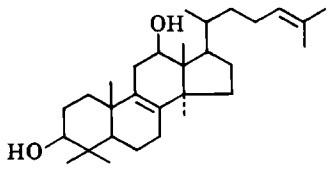
In an effort to elucidate the character of the double bond assumed to be in the side chain, obliquol was subjected to ozonolysis. The isolation of acetone from this treatment proved the presence of the isopropylidene group in the side chain.

Several oxidative procedures were attempted *in order* to locate the hydroxyl groups of obliquol. The Oppenauer oxidation, using various conditions, gave the same compound which, however, could not be thoroughly purified. The infrared spectrum indicated that this compound was an hydroxy ketone. The compound gave a positive Zimmermann test indicating a 3-keto group. The resistance of the second hydroxyl group to Oppenauer oxidation gave the first indication that obliquol contained two secondary hydroxyl groups, one of which was partially hindered.

Subsequent titration of obliquol with a standard acidic chromium trioxide solution confirmed that both hydroxyl groups were secondary since almost exactly two equivalents of oxidant were consumed, indicating the formation of two keto groups by this method.

Oxidation with chromium trioxide in acetic acid gave an impure product which showed no hydroxyl group absorption in the infrared but possessed a strong single band at 1715 cm.$^{-1}$, indicative of one or more keto groups in six-membered rings. This finding eliminated the five-membered D ring as the sight for the second oxygen function (2). Therefore, the positions that this second hydroxyl group could occupy were the 6, 7, 11, or 12. Position 1 could be eliminated since Oppenauer oxidation of a 1,3 dihydroxy system results in the loss of the 1-hydroxyl group (3). A 2-hydroxyl group was eliminated as a possibility since oxidation to an α-diketo system would be detected in the infrared spectrum.

The 7 or 11 hydroxyl group possibilities were eliminated since they would be allylic to the 8,9 double bond, hence quite susceptible to oxidation (4). The 6 α or 6 β hydroxyl groups have also been shown to be susceptible to Oppenauer oxidation (5). This narrowed the possibilities to the two epimeric 12 positions. In the case of the triterpene, polyporenic acid A, the 12 α hydroxyl group is sufficiently hindered to resist benzoylation (6). Obliquol formed a dibenzoate readily, which narrowed the possibilities to the 12 β position for the second hydroxyl group. This position is consistent with the findings but more conclusive evidence was derived from nuclear magnetic resonance data.



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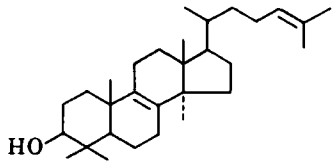

II

The resonances of obliquol (I), lanosterol (II), and their acylated products are discussed in turn, the numbers cited being tau values (see Fig. 1). Starting at low field there appears first the resonances of the olefinic hydrogens on the side chain as a broad triplet between 4.79 and 4.95. The exact value of the shift in obliquol diacetate, where another peak is superimposed on this one (the peak for the H on the same C as the acetoxy group) is in doubt. The H's on the same C as an OH group in the two triterpenes appear next. The C-3 H is at 6.69 and 6.80, while the other H on a C—OH in obliquol falls at 6.29. Acetylation shifts these peaks downfield by about 1.2 p.p.m. In obliquol diacetate they appear at 4.95 (nearly coincident with the olefinic hydrogen peak), and 5.43, and in lanosterol acetate at 5.52. The acetyl methyls appear at 7.85, 7.88, and 8.00. The two terminal methyls appear as a broadened pair of peaks between 8.3 and 8.5 in all compounds except obliquol diacetate. In this derivative there appears at this point in the spectrum a single broad hump with an area which corresponds to three methyl groups. We interpret this as the superposition on the pair of peaks of the isopropylidene methyls with a methyl group of which the chemical shift is reduced by the presence of the second hydroxyl group in obliquol. If this hydroxyl group is at the 12 position, then the nearest methyl group is the 18 methyl group. In corroboration of this, the C-18 peak appears in lanosterol at 9.32 and in lanosterol acetate at 9.35, but in obliquol and obliquol diacetate there are no methyl groups with chemical shifts this high.

The remaining methyl peaks cannot be definitely assigned. One of the peaks, that corresponding to the side chain methyl, should be a doublet. In lanosterol, half of the doublet can be seen at 9.10 and the other half is presumed to

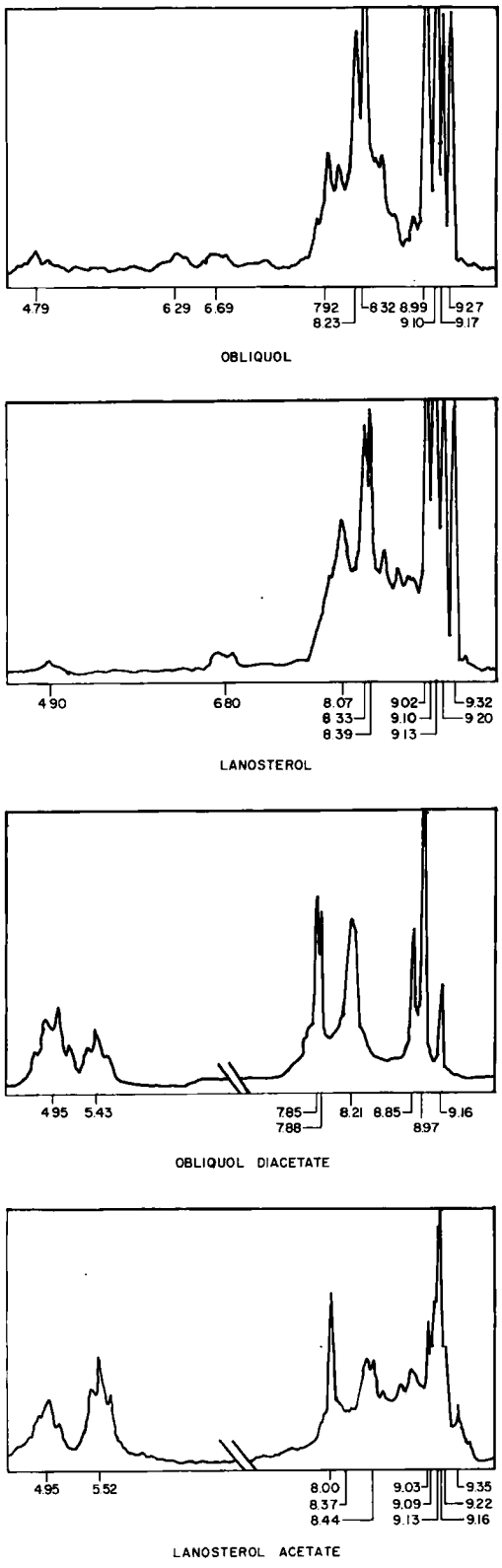

Fig. 1.—Nuclear magnetic resonance spectra of obliquol, lanosterol, and their acetylated products. Numbers are expressed as tau values.

be part of the peak at 9.02, the remainder of this peak corresponding to two other methyls, one of which is probably C-19. The remaining three ring methyl groups, at positions 4 and 14 are at 9.02 and 9.13.

In lanosterol acetate, the C-18 methyl group appears at 9.35, while one other methyl group is clearly defined at 9.03 and the remaining peaks are overlapping in the region surrounding 9.16. The principal effect of acetylation would be expected for the two methyls at position 4, and they seem to have been shifted upfield.

In obliquol, individual methyl peaks appear clearly defined at 9.10, 9.17, and 9.27. Two methyls appear in the resonance at 8.99, one of which is probably C-19. The doublet for the side chain methyl is apparently half in the peak at 8.99 and half in the peak at 9.10. In obliquol diacetate, that which is probably C-19 appears at 8.85, and what is probably the C-18 methyl group appears at 9.16. The remaining methyl groups appear in an unresolvable band at 8.97.

In consideration of the finding of the interference with the C-18 methyl hydrogens by one of the acetyl groups of obliquol diacetate, we conclude that this acetyl group is on the proximate C-12 position. Furthermore, from a study of a model this acetyl group in the 12 position must be β oriented in order to be in a position to interact with the β oriented C-18 methyl hydrogens. This finding was completely consistent with all of the chemical evidence and therefore led us to provisionally assign structure I to obliquol.

EXPERIMENTAL

Nuclear magnetic resonance spectra were determined with a Varian 4300-C spectrometer operating at 56.4 megacycles. The samples were examined as saturated solutions in deuterated chloroform. Chemical shifts were calculated from audio side bands applied by an oscillator with frequency continuously monitored by a Hewlett-Packard 523B counter. The shifts are expressed in tau values computed by using the H of the chloroform in the solvent as an internal reference and adding 2.72 p.p.m. to the shift observed from this reference. Several spectra were also referenced with external benzene, which falls 0.86 p.p.m. to high field of the chloroform peak. In the spectra of the acetates, the regions at low field were run at higher gain than the regions at high field.

Ozonolysis of Obliquol Diacetate.—One hundred milligrams of obliquol diacetate in 20 ml. of carbon tetrachloride was subjected to a stream of 3% ozonized oxygen at −5° for 30 minutes. The solution was then treated with water and powdered zinc and finally steam distilled into an acidic solution of 2,4-dinitrophenylhydrazine. The phenylhydrazone that formed was crystallized, m.p. 122–124°. A mixed melting point with authentic

acetone 2,4-dinitrophenylhydrazone gave no depression.

Oppenauer Oxidation of Obliquol.—A solution of 0.4 Gm. of obliquol in 5 ml. of cyclohexanone and 10 ml. of toluene was treated with 0.8 Gm. of aluminum isopropoxide and refluxed for 2 hours. This solution was cooled and 5 ml. of 10% sulfuric acid added. The solution was then extracted with ether and the residue recrystallized repeatedly from methanol, m.p. 160–165°. Further attempts to purify this compound were unsuccessful. The above procedure was repeated using aluminum tertiary butoxide. It was refluxed for 9 hours. A substance was obtained that was identical to the one obtained by the previous method. It also resisted further purification. Both compounds gave identical infrared spectra. In both cases the compounds gave positive Zimmermann tests but no absorption in the ultraviolet. The infrared spectra showed bands at 3450 cm.$^{-1}$ (O—H), 1715 cm.$^{-1}$ (keto carbonyl), and 1040 cm.$^{-1}$ (C—O).

Chromic Acid Titration of Obliquol.—Obliquol (221 mg., 0.0005 mole) was titrated with a chromium trioxide solution in sulfuric acid that was prepared according to the procedure described by Curtis (7) and Bowers (8). The volume of solution consumed was equivalent to the conversion of 1.95 hydroxyl groups to carbonyl groups.

Chromic Acid–Acetic Acid Oxidation of Obliquol.—A solution of 100 mg. of obliquol in 10 ml. of benzene was treated with 130 mg. of chromium trioxide in 1.5 ml. of water and 3 ml. of glacial acetic acid and allowed to stand for 18 hours. The benzene solution was separated, concentrated, and placed on an alumina column. A small fraction that was eluted with benzene-methanol remained as a viscous oil. The infrared spectrum showed no hydroxyl group absorption but showed a strong carbonyl peak at 1715 cm.$^{-1}$. The substance gave a positive Zimmermann test.

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New Method for Location of Organic Acids on Paper Chromatograms

By GILBERT C. SCHMIDT, CARL FISCHER†, and JOHN M. McOWEN

A modification of Riegler's nitrite test, employing betanaphthol, sodium nitrite, and naphthylamine or sulfanilamide, has been adapted for location of organic acids on paper chromatograms. Most of the twelve acids tested are detectable at a level of 5 mcg. or less although 50–75 mcg. are required in isolated cases. The reagent may be used with all four of the common solvent systems employed, with no notable differences in sensitivity. The sensitivities of this reagent and four previously described detection reagents have been compared. The new reagent is more sensitive than the best of the previously described procedures.

The introduction of paper chromatography as a method for identifying organic acids (1) has been followed by the use of numerous detection reagents. The acid-base indicators have been most frequently used, but colors are transient, complete solvent removal is essential, and it is difficult to distinguish true spots from artefacts. Better results have been reported if the indicator is added to the solvent system (2, 3), but this modification has not been generally accepted. Potassium permanganate, usually used to locate unsaturated and hydroxy compounds (4), has been combined with indicators to produce a general location reagent. The reported sensitivity is poor, but final colors and development times vary for different acids. The combined reagent has therefore been recommended to distinguish acids having similar R_f values (5).

The acid catalyzed condensation of aniline and reducing sugars to form colored compounds has been used to locate both sugars (6) and acids (7). Sugars are detected with an aniline-phosphoric acid mixture, while acids are detected with an aniline-xylose mixture. The aniline-xylose reagent and acridine appear to be the most useful of the remaining detection reagents (7), but both are carcinogenic and little has been written about them.

Nessler's reagent and starch-iodine-iodate reagent have been used to locate the ammonium salts of acids, but neither is a satisfactory general location reagent (7, 8). Aqueous ferric chloride

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acetone 2,4-dinitrophenylhydrazone gave no depression.

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The acid catalyzed condensation of aniline and reducing sugars to form colored compounds has been used to locate both sugars (6) and acids (7). Sugars are detected with an aniline-phosphoric acid mixture, while acids are detected with an aniline-xylose mixture. The aniline-xylose reagent and acridine appear to be the most useful of the remaining detection reagents (7), but both are carcinogenic and little has been written about them.

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may be used to locate hydroxy acids (4), but it is highly insensitive and is not generally applicable. A variety of diazonium salts are useful in selected instances, particularly for the detection of phenolic acids (8–10), but many metabolically important acids do not give colors. The same difficulty arises with ammonium vanadate, dinitrophenylhydrazine, *p*-dimethylaminobenzaldehyde, phosphomolybdic acid, potassium ferrocyanide-ferric ammonium sulfate, and dichlorophenolindophenol.

Chloranilic acid, previously used to detect inorganic ions and certain nitrogenous compounds, has been introduced as a highly sensitive reagent for locating sodium salts of organic acids (11). Volatile acids are stabilized and tailing is reduced, but buffered papers or chelating agents cannot be used. A neutral or basic solvent is required and the paper must be thoroughly dried to prevent interference by "ghost" spots.

Riegler's nitrite test (12) has been modified by the authors and introduced as a location reagent for acids (13). In Riegler's test, nitrites give a color with alphanaphthol and sodium naphthionate in acid solution. Acids give a positive test in the presence of alphanaphthol, sodium naphthionate, and sodium nitrite. Both tests result in a deep red color and apparently depend on the formation of an azo dye. We reasoned that paper chromatograms sprayed with a mixture of alphanaphthylamine and betanaphthol, followed by a sodium nitrite spray or dip, should form nitrous acid only in those spots occupied by migrated free acids. Consequently, only the acid spots should undergo diazotization, coupling, and color development.

The original method is highly sensitive and has been successfully used for more than a year, but replacement of the highly carcinogenic alphanaphthylamine is desirable. This report describes our original procedure and a recent modification in which sulfanilamide replaces the carcinogen. Sensitivities of these two procedures and some other available methods are compared.

EXPERIMENTAL

Standard Solutions.—Choice of reference acids was based on our own interests and on general biochemical importance. Commercial acids of the best available grade were used without further purification. Stock aqueous 1% solutions were prepared as needed. The quantity of acid applied to each spot was controlled by dilution of the stock solutions and by varying the sizes of micropipets throughout the range 1–25 μl.

Chromatogram Development.—Acids were chromatographed on Whatman No. 1 paper. Areas of applied spots were controlled by rate of sample delivery and by drying with unheated air from a hair dryer during application of the samples. Heat was avoided because some of the acids are unstable. Air dried chromatograms were then developed for the prescribed period by the ascending method.

Solvent Systems.—Several common solvent systems were chosen to determine if solvent composition would severely limit use of the alpha naphthylamine or sulfanilamide location reagents. Butanol-acetic acid-water (7), propanol-eucalyptol-formic acid-water (14), ethanol-ammonia-water (15), and an 8:1:1 propanol formic acid-water system were used for this purpose. The propanol-formic acid-water system was used for comparing sensitivities of the proposed reagents and several previously described reagents. Solvents were freshly prepared each day and were equilibrated for 1 hour before transferring to the chromatographic tanks. An additional 1 hour equilibration preceded introduction of the chromatograms. Chromatograms were routinely dried at room temperature for at least 4 hours, but in most instances location reagents could be applied after drying for 1 hour.

Alphanaphthylamine Reagent.—Chromatograms were sprayed with a solution of alphanaphthylamine (1%) and betanaphthol (0.5%) in 95% ethanol. They were then air dried at room temperature until the ethanol had evaporated, but for no longer than 1 hour. Dried chromatograms were then sprayed with 1% aqueous sodium nitrite. Acids immediately appear as dark orange spots on a pale pink background. Intensity of the background color increases on standing but is less pronounced if the naphthylamine-naphthol solution is allowed to age for a day or two before use. This solution is stable for at least two weeks at room temperature, but the sodium nitrite solution is preferably prepared just before use since it is stable for only 12 hours at room temperature.

Sulfanilamide Reagent.—Chromatograms were treated as described for alphanaphthylamine reagent, except that the initial spray contained 2% sulfanilamide and 0.5% betanaphthol in 95% ethanol. Following the sodium nitrite spray, acids immediately appear as orange spots on an almost colorless background. Background color does not appreciably increase when chromatograms are stored for several weeks. The use of aged reagent has no advantage.

Other Location Reagents.—Four previously described location reagents were chosen for sensitivity comparisons. Aniline-xylose (7), acridine (7), and combined indicator-permanganate reagent (5) were prepared and used as previously described. Bromcresol green reagent (16) was purchased in spray cans.[1]

RESULTS AND DISCUSSION

Preliminary Experiments.—Filter paper circles were spotted with 10 μl. quantities of tropic acid solutions, representing different amounts of acid. These test plates were then developed under a variety of experimental conditions to establish the feasibility, potential sensitivity, and certain requirements for color development.

[1] "Spraytec" reagents, available from Aloe Scientific Co., St. Louis, Mo.

In support of the assumption that diazotization and coupling are responsible for color development, a variety of diazotizable amines and a variety of coupling phenols or amines produced colors. Of the combinations tested, the naphthylamine-naphthol and sulfanilamide-naphthol reagents gave the best results. Only these combinations were further studied.

These experiments also disclosed that the diazotizable amine and the coupling agent must be applied as a single solution, that the sodium nitrite must be freshly prepared to prevent intense coloration of the entire chromatogram, that the use of ice cold reagents decreases sensitivity, and that application of reagents by spraying is preferable to dipping.

Sensitivity of the Proposed Method.—Preliminary estimates of sensitivity were obtained from the previously described spot plates, but these tests were confined to tropic and mandelic acids and did not involve migration. Accurate sensitivity comparisons were therefore conducted under more probable experimental conditions.

Twelve commonly encountered aliphatic acids, and the propanol-formic acid-water system were arbitrarily chosen. Graded amounts of each acid were applied to a series of chromatograms. The chromatograms were then developed and were sprayed with naphthylamine or sulfanilamide reagent as previously described. Sensitivities shown in Table I represent the lowest levels at which color could be visually detected when chromatograms were held against a window and viewed by daylight. At levels somewhat higher than those recorded, spots were immediately visible after spraying with sodium nitrite.

TABLE I.—SENSITIVITIES OF NAPHTHYLAMINE AND SULFANILAMIDE REAGENTS

Acids Tested[a]	Mcg. of Acid Detected per 10 μl. Spot: Sulfanilamide	Mcg. of Acid Detected per 10 μl. Spot: Naphthylamine
Citric	2	7
Lactic	2	4
Succinic	2	3
Malic	2	5
Malonic	2	6
Tropic	2	3
Mandelic	3	2.5
cis-Aconitic	5	70
Oxalacetic	40	60
Glyoxylic	2	1
Tartaric	5	9
α-Ketoglutaric	2	4

[a] All chromatograms were developed in propanol-formic-water.

The reported sensitivities were further checked by chromatographing mixtures of acids in propanol-water-formic acid. Mixtures were prepared as stock solutions containing 50 mcg. of each component in 10 μl. of solution. Each mixture, and dilutions of it, were spotted on a single chromatogram which was then developed and sprayed as previously described. Figure 1 shows a representative chromatogram. It is apparent that the individual components of a mixture are visible in a photograph when the concentrations are approximately twice the minimal detectable levels shown in Table I. The same situation occurs with chromatograms of single acids, consequently sensitivities are not appreciably altered when a mixture is separated.

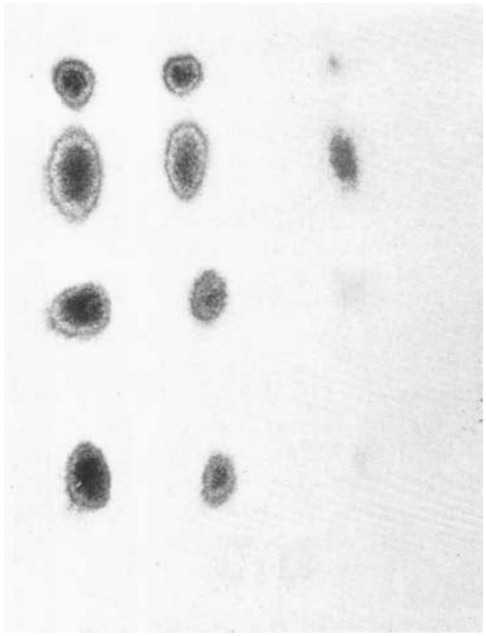

Fig. 1.—Representative separation of acid mixture at different concentration levels: solvent, propanol-formic acid-water (8:1:1); acids in order of increasing R_f value, tartaric, malic, succinic, and mandelic; acid concentrations, from left to right, 50, 25, and 10 mcg. of each component.

Although this report is not primarily concerned with separation techniques, it may be noted that the propanol-formic acid-water system produces excellent separation of many acids. Spots are compact and there is no tailing.

Comparative Sensitivities.—Sensitivities of the naphthylamine and sulfanilamide reagents were compared with four previously described general acid location reagents. For the sake of simplicity, all comparisons were made after migration in the propanol-formic acid-water system. Sensitivities of the previously described reagents for the twelve acids used in this study are shown in Table II. The data in Table II provide a controlled comparison of these four methods and, in conjunction with data in Table I, illustrate the excellent sensitivity of the new reagents.

In obtaining these data, it was noted that the degree of increased sensitivity obtained by reading acridine stained chromatograms by ultraviolet light is extremely time dependent. The more quickly chromatograms are read, the greater is the increase in sensitivity. Tabulated values were obtained by reading immediately after dipping. The sensitivity of the combined indicator-permanganate reagent of Paskova and Munk was confirmed, but we were unable to obtain the color variations reported by these authors.

Effect of Solvent Composition on Sensitivity.—Since the requisite use of a single solvent system would severely limit usefulness of the proposed reagents, the effects of four common solvent systems were investigated. The sensitivity of both alpha-

TABLE II.—SENSITIVITIES OF SELECTED DETECTION REAGENTS

Acids Tested[a]	Mcg. Acid Detected per 10 μl. Spot: Paskova Munk	Aniline Xylose	Bromcresol Green	Acridine	Acridine (u.v.)
Citric	10–50	5	5	50	20
Lactic	60	15	8	150	20
Succinic	50	8	15	20	15
Malic	125	8	10	50	15
Malonic	30	5	8	20	10
Tropic	30	5	5	No spot	No spot
Mandelic	80	10	15	50	20
cis-Aconitic	125	10	3	50	10
Oxalacetic	>150	>50	20	100	15
Glyoxylic	50	5	25	100	20
Tartaric	80	5	15	20	15
α-Ketoglutaric	50	4	8	20	10

[a] All chromatograms developed in propanol-formic-water.

TABLE III.—SENSITIVITY OF NAPHTHYLAMINE REAGENT USED AFTER DIFFERENT DEVELOPING SOLVENTS

Acids Tested	Mcg. Acid Detected per 10 μl. Spot: Propanol Formic Water	Butanol Acetic Water	Ethanol Ammonia	Propanol Formic Eucalyptol
Citric	7	7	5	10
Lactic	4	7	8	8
Succinic	3	3	3	5
Malic	5	5	6	9
Malonic	6	7	7	10
Tropic	3	3	3	5
Mandelic	2.5	3	3.5	5
cis-Aconitic	70	80	55	70
Oxalacetic	60	55	No spot	70
Glyoxylic	1	2	3	10
Tartaric	9	9	12	10
α-Ketoglutaric	4	4	4	7

TABLE IV.—SENSITIVITY OF SULFANILAMIDE REAGENT USED AFTER DIFFERENT DEVELOPING SOLVENTS

Acids Tested	Mcg. Acid Detected per 10 μl. Spot: Propanol Formic Water	Butanol Acetic Water	Ethanol Ammonia	Propanol Formic Eucalyptol
Citric	2	6	9	2
Lactic	2	8	10	4
Succinic	2	1	4	1
Malic	2	4	8	3
Malonic	2	8	6	2
Tropic	2	1	9	4
Mandelic	3	6	10	5
cis-Aconitic	5	No spot	9	10
Oxalacetic	4.0	30	60	20
Glyoxylic	2	1	1	1
Tartaric	5	8	10	9
α-Ketoglutaric	2	9	12	2

naphthylamine reagent and sulfanilamide reagent was determined for each of the twelve reference acids in each solvent system, in the manner described above. The results, shown in Table III and Table IV, demonstrate that none of the four solvents would prohibit use of the proposed reagents. Both reagents could be used with any of these systems, and both may be directly applied to chromatograms that have been developed with ammoniacal solvents. The effect of buffered papers is unknown, since no attempt was made to evaluate this variable.

The failure to obtain a spot with sulfanilamide reagent in the presence of 100 mcg. of *cis*-aconitic acid, using butanol-acetic acid-water as the developing solvent, is unexplained. The same is true for naphthylamine reagent and oxalacetic acid in the ethanol-ammonia system. It should also be noted that some spots may develop slowly with the sulfanilamide reagent. Chromatograms sprayed with this reagent should therefore be re-examined for latent spots after standing overnight.

SUMMARY AND CONCLUSIONS

Two new reagents have been developed for the location of organic acids on paper chromatograms. Color development apparently depends on an acid catalyzed diazotization and coupling to form a highly colored compound, since colors are produced in the presence of a wide variety of diazotizable amines and coupling amines or phenols. The proposed reagents differ in the nature of the diazotizable amine, one employing naphthylamine and the

other sulfanilamide. The sulfanilamide reagent is generally, but not universally, the more sensitive. It usually produces less background color than naphthylamine and the spots are more stable because the background darkens much less rapidly. The sulfanilamide reagent does not have the disagreeable odor of naphthylamine and does not have the carcinogenic action of the latter compound.

Both reagents have been tested against twelve reference acids in four commonly used solvent systems. Sensitivities vary somewhat from one solvent to the next, but the variations are minor and do not prevent use of any solvent tested. Both reagents are much more sensitive than the indicator-permanganate or acridine procedures. The sulfanilamide reagent is notably more sensitive than aniline-xylose or bromcresol green and does not possess the carcinogenicity of aniline-xylose. Both reagents give more reproducible sensitivities than any reagent tested, and neither produces "ghost" spots.

Intense background colors may develop with either sulfanilamide or alphanaphthylamine if chromatograms are exposed to high concentrations of laboratory fumes, but minimal care will prevent this occurrence.

An 8:1:1 propanol-formic acid-water system has been used to develop most of these chromatograms. It has not been extensively studied as a solvent for separating organic acids, but may certainly be used to advantage. It produces excellent separations of many compounds tested, causes little or no tailing, and gives compact spots.

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Protective Coatings XVI

Disintegration of Protective-Coated Tablets as Determined by Urinary Excretion in Humans

By TADAO IDA, SHOJI TAKAHASHI, KAZUO NODA, SHUZO KISHI, SETSUO NAKAGAMI, and ISAMU UTSUMI

A disintegration test of protective coated preparations in human bodies was carried out by determining the riboflavin amount excreted in urine after administration of the riboflavin tablets coated with the previously reported protective-coating agents. Though the rate of the riboflavin excretion was somewhat slow, there was no indication of unusual disintegration of the coated tablets in human bodies.

IN PRECEDING PAPERS, studies on the protective-coating agents were reported in which the amino derivatives and the amino acid derivatives of cellulose, saccharides and polyhydric alcohols, polyvinylamines, polyvinylaminoacetals, polyvinylpyridines, and others were synthesized and examined (1–12). Polyampholites of the vinylpyridine-methacrylic acid system were also studied for the protective-coating agents which solubilize in both gastric and intestinal juice (13). All preparations coated with these agents showed excellent results in the tests—water-resistance, *in vitro* disintegration, and others.

In this report, the *in vivo* test was examined in human bodies for the disintegration of the preparations. Since riboflavin absorbed in excess is rapidly excreted in urine, disintegration rate was determined by the riboflavin amount in urine excretion after the administration of the coated riboflavin tablets.

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Protective Coatings XVI

Disintegration of Protective-Coated Tablets as Determined by Urinary Excretion in Humans

By TADAO IDA, SHOJI TAKAHASHI, KAZUO NODA, SHUZO KISHI, SETSUO NAKAGAMI, and ISAMU UTSUMI

A disintegration test of protective coated preparations in human bodies was carried out by determining the riboflavin amount excreted in urine after administration of the riboflavin tablets coated with the previously reported protective-coating agents. Though the rate of the riboflavin excretion was somewhat slow, there was no indication of unusual disintegration of the coated tablets in human bodies.

IN PRECEDING PAPERS, studies on the protective-coating agents were reported in which the amino derivatives and the amino acid derivatives of cellulose, saccharides and polyhydric alcohols, polyvinylamines, polyvinylaminoacetals, polyvinylpyridines, and others were synthesized and examined (1–12). Polyampholites of the vinylpyridine-methacrylic acid system were also studied for the protective-coating agents which solubilize in both gastric and intestinal juice (13). All preparations coated with these agents showed excellent results in the tests—water-resistance, *in vitro* disintegration, and others.

In this report, the *in vivo* test was examined in human bodies for the disintegration of the preparations. Since riboflavin absorbed in excess is rapidly excreted in urine, disintegration rate was determined by the riboflavin amount in urine excretion after the administration of the coated riboflavin tablets.

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EXPERIMENTAL

Tablets.—The compressed tablets (9.0 mg. of riboflavin each) were used in the experiments. Mean thickness, weight, and diameter of the tablets were 3.5 mm., 120 mg., and 7 mm., respectively. The disintegration time of the tablets in distilled water, artificial gastric juice, and artificial intestinal juice was 2–3 minutes as determined by the method specified in U.S.P. XVI (14).

Protective-Coated Tablets.—By the dipping method, the compressed riboflavin tablets were coated with two different coating solutions: 10% methanol solution of the copolymer, 2-vinyl-5-ethylpyridine-styrene (VEP-St), and 10% ethanol-trichloroethane (1:1) solution of the copolymer, 2-vinyl-5-ethylpyridine-methacrylic acid-methylacrylate (VEP-MAA-MA).

TABLE I.—*In Vitro* DISINTEGRATION TEST OF THE COATED TABLETS

Tablets	Film Thickness, μ	Disintegration Time: Distilled Water, hrs.	Disintegration Time: Artificial Gastric Juice, min.	Disintegration Time: Artificial Intestinal Juice
VEP-St-coated	150	4	5–10	4 hrs.
VEP-MAA-MA-coated	145	4	5–10	10–15 min.

Mean thicknesses of the coating films and disintegration times of the coated tablets are shown in Table I. The mean thicknesses were calculated from the mean weights of coating agents applied to each tablet. The disintegration time of the coated tablets in distilled water, artificial gastric juice, and artificial intestinal juice was determined by the method specified in U.S.P. XVI (14).

Administration test.—Seventy-nine men and 52 women ranging from 18–55 years were divided into three groups, A, B, and C (as shown in Table II), and each person was administered one tablet—uncoated tablets for the A group, VEP-St-coated tablets for the B group and VEP-MAA-MA-coated tablets for the C group. The amounts of riboflavin in total urine excreted in 3 hours prior, 3 hours, and 6 hours after administration were determined individually by the U.S.P. fluorometric procedure (14).

TABLE II.—GROUPS IN ADMINISTRATION

Group	Administered Tablet	Men	Women	Total
A	Uncoated	20	10	30
B	VEP-St-coated	29	21	50
C	VEP-MAA-MA-coated	30	21	51

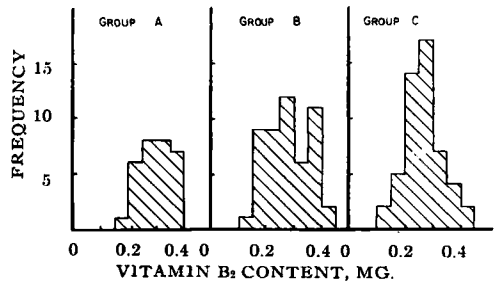

Fig. 1.—Vitamin B_2 content of urine before administration.

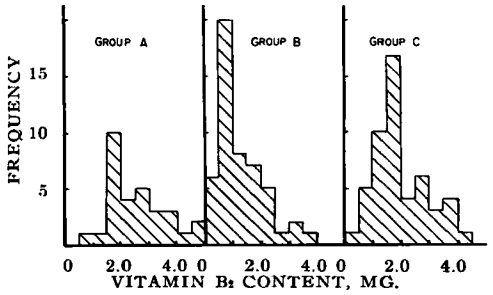

Fig. 2.—Vitamin B_2 content of urine 0–3 hours after administration.

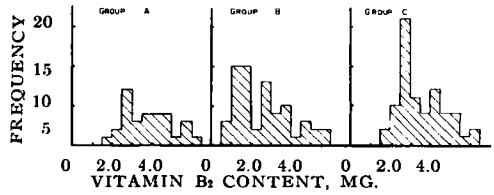

Fig. 3.—Vitamin B_2 content of urine 0–6 hours after administration.

RESULTS AND DISCUSSION

Riboflavin Contents of Urine Before Administration.—Figure 1 shows the riboflavin content of urine before administration. All individuals showed almost similar values, and no particular difference was observed between men and women.

Amounts of Riboflavin Excreted in Urine After Administration.—Figures 2 and 3 show the total amounts of riboflavin excreted in urine during 3- and 6-hour periods after administration, and Table III shows the mean values of riboflavin in the three groups after 6 hours.

As shown in Table III, the B group excreted less riboflavin than the other two groups, and women excreted less than men. Analysis of the variances by Snedecor's method (15) shows that these differences are statistically significant at the 5% level, but

TABLE III.—MEAN AMOUNT OF RIBOFLAVIN EXCRETED IN URINE IN 0–6 HOURS

Tablets	Men: Administered Number	Men: Mean Value of Excreted Riboflavin, mg.	Women: Administered Number	Women: Mean Value of Excreted Riboflavin, mg.	Mean: Administered Number	Mean: Mean Value of Excreted Riboflavin, mg.
A	20	3.95	10	3.49	30	3.797
B	29	2.87	21	2.26	50	2.614
C	30	3.60	21	3.24	51	3.447
Total	79	3.40	52	2.89	131	3.211

the lower excretion of women might result from some errors in urine collection.

Variation of Riboflavin Amounts Excreted in Urine With the Lapse of Time.—Table IV shows the mean values of riboflavin excreted in both 3-hour periods. X_1 and X_2 indicate milligrams of riboflavin excreted in 3 hours and the next 3 hours, respectively.

TABLE IV.—MEAN VALUES OF RIBOFLAVIN EXCRETED IN 0–3 (X_1) AND 3–6 (X_2) HOURS

Tablet Group	X_1, mg.	X_2, mg.
A	2.512	1.285
B	1.251	1.363
C	1.951	1.496

The B and C groups showed lower values of X_1 as compared with the A group. But the C group showed the highest X_2 value of the three, and the total for 6 hours was nearly the same as the A group.

The B group (as described above) showed the lowest total value of the three, but the X_2 value was greater than the X_1. It can be seen that the excretion gradually increased with the lapse of time, and further increase of the value might be expected in the following period.

These differences of riboflavin excretion between the groups were statistically significant at the 5% level in the result of analysis of dispersion.

The results show that the excretion of riboflavin with the coated tablets was slightly slower than the uncoated tablets. This was expected as the disintegration rate of the coated tablets was somewhat retarded.

Disintegration of Coating Films in Humans.—Variances of the amounts of excreted riboflavin in each group were calculated in order to see whether an unusual disintegration of the coated tablets occurred.

TABLE V.—VARIANCES OF RIBOFLAVIN EXCRETED IN 0–3 AND 0–6 HOURS

Tablet Group	0–3 Hours	0–6 Hours
A	1.0220	1.3257
B	0.7204	1.9221
C	0.8080	1.6065

As can be seen in Table V, amounts of the excretions showed no significant difference between three groups and, it can be considered that there were no unusual disintegrations in humans with both coated tablets.

It may be concluded from these results that the coated tablets must disintegrate to release medicaments in humans.

SUMMARY

1. Riboflavin tablets were coated with the copolymers 2-vinyl-5-ethylpyridine-styrene (VEP-St), and 2-vinyl-5-ethylpyridine-methacrylic acid-methyl acrylate (VEP-MAA-MA). The disintegration of the coated tablets was tested by determining the riboflavin amount excreted in human urine after administration of the tablets.

2. The group administered VEP-St-coated tablets showed lower excretion of riboflavin in 6 hours after administration than the group administered uncoated tablets. The group administered VEP-MAA-MA coated tablets showed nearly the same as the control in the excretion.

3. Both groups administered the coated tablets showed lower excretions of riboflavin in 3 hours after administration, and higher excretions in the following 3 hours than the control.

4. The coated tablets disintegrated in humans to release medicaments. Any unusual disintegration did not occur.

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Relationship of Prostatic Serum Acid Phosphatase to Age and Size of the Prostate in Rats

By R. H. WOJNAS† and J. F. BESTER

Serum of freshly killed rats of various ages was treated with *p*-nitrophenyl phosphate in citric acid buffer solution pH 4.8. Total serum acid phosphatase was determined colorimetrically on the basis of *p*-nitrophenol formation. Residual serum acid phosphatase was determined on duplicate serum specimens similarly, but with the addition of L (+) tartrate. Prostatic serum acid phosphatase activity was computed by subtraction. This activity was found to increase markedly until prostate weight reached approximately 200 mg. after which there was little change in activity. Increases in prostatic weight continued as body weight and age increased. Hence, in young animals prostatic serum acid phosphatase activity is indicative of normal prostatic growth and not necessarily indicative of abnormality.

PROSTATIC enlargement is one of the more common afflictions of the elderly male, occurring in from 30–40 per cent of men over 60 years of age. In general, such enlargement is due to benign hypertrophy, carcinoma, or fibrosis. Despite the fact that the resulting symptomology, if any, will show many similarities regardless of cause, it is obviously important that early differential diagnosis be made so that appropriate corrective measures may be undertaken.

As early as 1935 Kutscher and Wolbergs (1) reported that normal prostatic tissue is extraordinarily rich in a phosphatase having an optimum activity at about pH 5.0. It is present principally in the Golgi region of prostatic epithelium, while a second prostatic phosphatase with an optimum activity at about pH 10.5 is found in the basement membrane of the acini of the gland (2). The former, with optimum activity at pH 4.9, has been designated "acid" phosphatase; the latter has been designated "alkaline" phosphatase.

Significant amounts of acid phosphatase appear in blood plasma as a result of malignant growth of the prostate and metastases of prostatic cancer in the bones (3). It has been shown that prostatic acid phosphatase can enter the circulation very readily (4). Gutman, Sproul, and Gutman (5) noted the presence of acid phosphatase at the site of skeletal metastases secondary to carcinoma of the prostate. Huggins and Hodges (6) observed marked increases in serum concentration of acid phosphatase in prostatic carcinoma. They reported that the concentration could be decreased by reducing the activity of androgens by castration or by estrogen administration and increased by the injection of androgens. Gutman and Gutman (7) reported that serum acid phosphatase activity exceeded maximal normal values in 12 of 15 cases of disseminated prostatic carcinoma, but that there was no significant increase in such prostatic diseases as benign hypertrophy and acute prostatitis, nor in uremia or lymphosarcoma.

Because of observations such as these, the determination of serum levels of prostatic acid phosphatase has become a routine step in the diagnosis of prostatic cancer. Indeed, Bonner, *et al.* (8), discovered the presence of unsuspected prostatic carcinomas in five patients by this technique. They also reported that in three cases diagnoses of prostatic cancer based upon detection of nodules by finger palpation were

TABLE I.—BODY WEIGHT, PROSTATE WEIGHT, AND PROSTATIC SERUM ACID PHOSPHATASE ACTIVITY

Log Body Weight Gm. × 1000	Log Enzyme[a] Activity O.D. × 1000	Log Prostate Weight Gm. × 1000
5.704	2.188	2.542
5.688	2.215	2.427
5.664	2.387	2.762
5.661	2.270	2.708
5.652	2.241	2.971
5.652	2.428	2.185
5.647	1.973	2.348
5.644	2.215	2.707
5.644	2.366	2.772
5.631	2.260	2.697
5.615	2.164	2.603
5.580	2.310	2.430
5.185	2.649	1.398
5.121	2.808	1.301
5.093	2.573	1.114
5.090	2.742	1.398
5.079	2.792	1.176
5.079	2.845	0.699
5.072	2.937	1.230
5.068	2.822	1.000
5.068	2.887	1.079
5.065	2.438	1.230
5.041	2.640	1.362

[a] Activity expressed as optical density of solution.

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† Present address: School of Medicine, University of California, San Francisco.

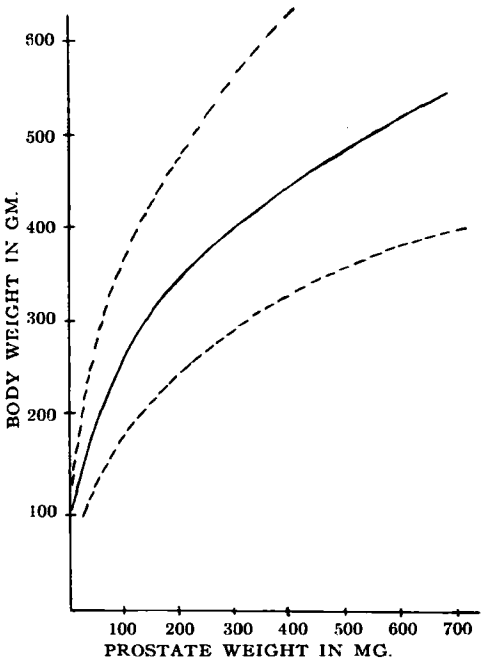

Fig. 1.—Relationship of body weight and prostate weight, showing mean values and 90% confidence limits.

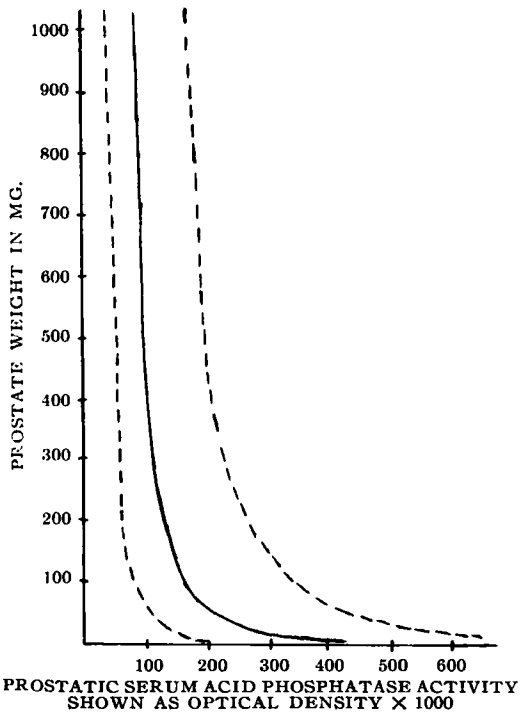

Fig. 2.—Relationship of prostate weight and prostatic serum acid phosphatase activity, showing mean values and 90% confidence limits.

shown to be incorrect by this test; the error was further shown by subsequent biopsy indicating benign hypertrophy.

Earlier work in our laboratory indicated that in some respects the prostate was markedly influenced by age (9). It therefore seemed important to determine whether prostatic serum acid phosphatase level and activity change appreciably at various age levels.

Various investigators have used *p*-nitrophenyl phosphate as a reagent for measuring phosphatase activity (10, 11). It is colorless, but the *p*-nitrophenol liberated by phosphatase hydrolysis is yellow and has a maximum absorbance between 400–420 mμ. The substrate is thus the indicator in the test. However, it is not specific for prostatic acid phosphatase in serum. As reported by Fishman and Lerner (12), erythrocytes increase serum phosphatase levels upon hemolysis. The same investigators showed that L-tartrate will inhibit prostatic serum acid phosphatase but will not inhibit other serum phosphatases. Thus, it is possible to determine total serum phosphatase activity of a specimen and then, by L-tartrate inhibition, to determine "residual" phosphatase activity. The difference between these will be prostatic serum acid phosphatase activity. This procedure formed the basis of the method used in the study being reported.

EXPERIMENTAL

Equipment and Reagents.—Wistar strain rats were used in the study. No restrictions in diet were observed. The rats were of two general age groups—about 6 weeks and about 18 months. The *p*-nitrophenyl phosphate reagent and the buffer solutions were obtained commercially as a prepared kit for phosphatase determination.[1] All other reagents used were Baker and Adamson reagent grade chemicals. Colorimetric determinations were made in a Beckman DU spectrophotometer.

Procedure.—After recording the age and weight of the rat, it was sacrificed in a closed jar of ether. An incision was made along the ventral midline of the body and 5–10 ml. of blood were removed from the aorta with a syringe. The blood was centrifuged in a refrigerated clinical centrifuge for approximately 10 minutes or until complete separation of serum had been obtained. The serum was then removed by pipet and frozen until used in the determination of its enzyme content.

The viscera of the rat were then pushed to one side and the prostate gland was teased from its surrounding organs and fat deposits. The lobes of the prostate were excised with a minimum of connective tissue. The gland was placed in a refrigerated calcium chloride desiccator overnight. The following day the gland was weighed and assayed for its total iodine content by the method of Menschenfreund (13). This procedure was dictated by the concurrent study (in this laboratory) of the

[1] Sigma Chemical Co., St. Louis, Mo.

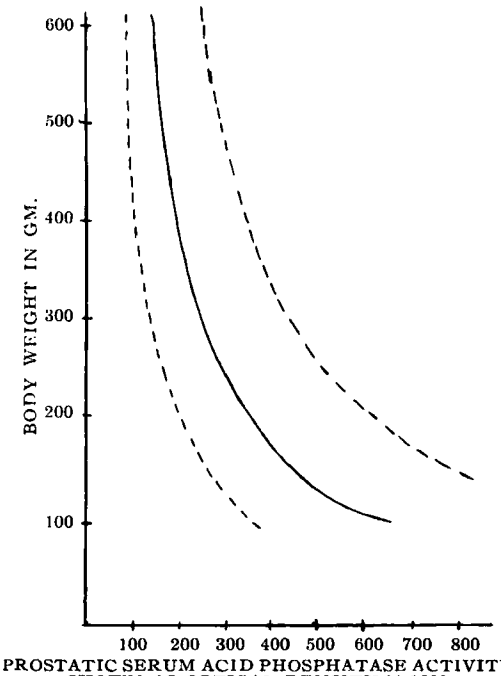

Fig. 3.—Relationship of body weight and prostatic serum acid phosphatase activity, showing mean values and 90% confidence limits.

possible role of iodine in prostatic function. However, this study is not part of the present report.

Total serum acid phosphatase and residual serum acid phosphatase activities were determined by standard procedures (14). The activity of prostatic serum acid phosphatase was obtained by subtraction.

It was unnecessary to prepare a standard curve for the Beckman DU spectrophotometer since relative rather than absolute values for phosphatase activity were being studied.

RESULTS AND DISCUSSION

Data obtained in the study appear in Table I. Logarithmic transformation of the data was necessary to reduce skewness. In all cases the data were multiplied by 1000 before determining their respective logarithms in order to avoid the use of negative computations.

Ages of the rats are not shown. Obviously, animals of approximately 18 months of age were much heavier than those of approximately 6 weeks of age. From examination of Table I, it is apparent that age is paralleled by body weight with an evident separation between the groups.

The data in Table I were then transformed to their respective arithmetic values; these were then used to show the relationships of interest in Figs. 1, 2, and 3.

The relationship between body weight and prostate weight shown in Fig. 1 has a t value of 15.9 with $p < 0.001$. As expected the greatest rate of increase in prostatic weight occurred in animals whose body weights were less than 200 Gm.

Figure 2 indicates the relationship between prostatic serum acid phosphatase activity and prostate weight. Here the t value was 6.64 with $p < 0.001$. This is rather remarkable correlation, and as Fig. 2 clearly shows, enzyme activity is greatest in those rats whose prostates weigh less than 200 mg., *i.e.*, in young rats. This is also indicated by Fig. 3; enzyme activity is markedly greater in young rats with body weights of less than 200 Gm. In this latter relationship, a t value of 8.59 with $p < 0.001$ was obtained.

Elevation of prostatic serum acid phosphatase activity has been accepted as an indication of prostatic cancer. Results of this study indicate that care must be exercised to prevent incorrect interpretation of the test in the young because activity of that enzyme is considerably higher in young animals.

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Preparation of Certain Biologically Active Anthraquinone Derivatives Containing Nitrogen

By ALFRED C. CORE† and ERNST R. KIRCH

A new approach to the synthesis of benzo[g]quinoline-5,10-diones is presented and discussed. Five benzo[g]quinoline-5,10-diones and two amino-hydroxyanthraquinones were prepared and screened for antibacterial activity. Some inhibition of growth of one or more common organisms was demonstrated by most of the compounds tested.

SINCE SOME naturally occurring hydroxyanthraquinones as well as other compounds of similar structure have been shown to possess antibacterial activity (1–3), it was felt that a series of such compounds might be tested for like activity. In addition, since many of the known antibiotics contain nitrogen, either in the form of an amine or within one of the rings of the compound (as in the actinomycins), it was desired to investigate a series of benzo[g]quinoline-5,10-diones for similar activity.

For this purpose the compounds (hitherto not synthesized) prepared and screened for antibacterial activity were 6,9-dihydroxybenzo[g]-quinoline-5,10-dione(I), 6-hydroxy-9-chlorobenzo[g]quinoline-5,10-dione(II), 6,9-dichlorobenzo[g]quinoline-5,10-dione(III), 1-hydroxy-4-dimethylaminoanthraquinone(IV), bis-(4-chlorophenyl) quinolinate(V).

It was desired to have a compound which had been prepared previously and which could serve as a reference compound for comparison of physical and chemical properties of the new compounds. The particular reference compound chosen was 2-hydroxybenzo[g]quinoline-5,10-dione-4-carboxylic acid, which was prepared from 3-amino-2-naphthoic acid according to the method of Etienne and Staehelin (4, 5). As reported by these authors, the compound when heated decarboxylated to form 2-hydroxybenzo[g]quinoline-5,10-dione, which in turn sublimed.

Since such treatment led to appreciable decomposition of the compound with resulting loss of material, it was decided to carry out the decarboxylation under reduced pressure.

However, when the material was heated *in vacuo*, the product collected was not the expected decarboxylated compound. Instead it was found that at low pressure the carboxylic acid could be sublimed unchanged.

No attempt was made to synthesize compounds bearing substituents on the benzenoid ring by this method, since the yields for all steps after formation of 1-acetylbenzindoxyl were good only if the quantities of starting material were kept within a range of from 100–500 mg.

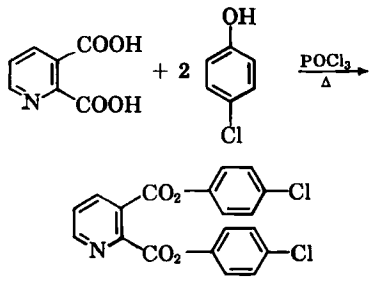
As an alternative it was hoped that a method might be found for condensation of quinolinic acid, its acid chloride or anhydride with a suitably substituted aromatic nucleus.

Attempts to condense the acid or its derivatives with activated aromatic ring systems using aluminum chloride (6), polyphosphoric acid (7), or sulfuric acid (8), were unsuccessful. No tractable products other than starting material were obtained.

Phosphoryl chloride acts as a Lewis acid which might serve to promote the formation of the desired benzo[g]quinolinediones. Further support for its use lay in the finding that aged phosphoryl chloride usually contains polyphosphoric acid (9) which might be expected to further the cyclization.

Treatment of *p*-chlorophenol and quinolinic acid with phosphoryl chloride resulted in the formation of a product which proved to be bis-(4-chlorophenyl) quinolinate.

Formation of the ester may be represented by



The use of a combination of phosphoryl chloride and polyphosphoric acid has been reported (10, 11). Assuming polyphosphoric acid could arise only from hydrolysis of the

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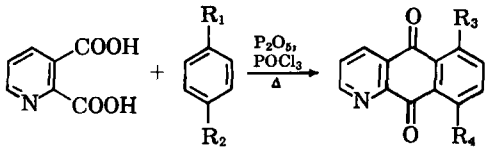
phosphoryl chloride in aged samples of the latter, phosphorus pentoxide should react with any phosphoric acid—arising either from hydrolysis or as the result of reaction with the quinolinic acid—to produce polyphosphoric acid.

Treatment of a 2.5:1 molar ratio mixture of quinolinic acid and hydroquinone monomethyl ether with the mixture of phosphoryl chloride and phosphorus pentoxide under reflux conditions led to the formation of 6,9-dihydroxybenzo[g]-quinoline-5,10-dione (I). This same method (with but slight modifications) was used in the preparation of the other benzo[g]quinoline-5,10-diones.

A general equation for these syntheses is



I. R_1 = —OCH_3; R_2 = —OH, —OCH_3; R_3 = R_4 = —OH
II. R_1 = R_3 = —OH; R_2 = R_4 = —Cl
III. R_1 = R_2 = R_3 = R_4 = —Cl

When the same reaction was run using the dimethyl ether of hydroquinone in place of the monomethyl ether an identical product was formed, as confirmed by physical constants and mixed melting point determination. Product identity was confirmed because the ether underwent cleavage to the phenolic compound when treated with phosphoryl chloride under reflux conditions. That cleavage occurred after ring closure was suggested by the somewhat better product yield when the dimethyl ether was used. Such an increase in yield could be attributed to the greater stability toward oxidation of the ether, hydroquinone itself tending to undergo oxidation to the quinone, which would be expected to be less amenable to attack by the electrophilic agent.

Treatment of *p*-chlorophenol and quinolinic acid under the same conditions led to the formation of a mixture of products. Since more than 94% of the total yield consisted of one isomer, it was felt that this compound was 6-hydroxy-9-chlorobenzo[g]quinoline-5,10-dione. Such an assumption was made on the basis that such reactions as have been reported which involve a Friedel-Crafts reaction between quinolinic acid and a benzenoid nucleus have led only to the formation of substituted picolinic acids rather than nicotinic acids (6); and further, that reactions involving phthalic acid condensations with substituted phenols to form substituted benzoylbenzoic acids have produced reactions *ortho* to the hydroxyl group when *p*-chlorophenol, *o*-cresol, or *p*-cresol were used (12).

It was possible to synthesize the 6,9-dichloro compound by the use of *p*-dichlorobenzene. In this case the yield was somewhat lower than when phenolic compounds were used. This is not surprising when one considers that the latter are in general much more amenable to nucleophilic attack.

BIOLOGICAL TESTING[1]

Due to the similarity in structure to some of the antibiotics currently in use, it was hoped that the compounds which were prepared might show some bacteriostatic activity. With this in mind, it was decided to screen the compounds for activity against several common organisms.

Since a rapid screening was desired, it was felt that a method similar to the original disk method (13) might well be used. Although the procedure was not quantitative, some quantitation could be achieved by comparison of the effects produced by the test compound with those of a known antibiotic.

A technique was employed whereby small filter paper disks were impregnated with alcoholic solutions of the compounds, and after streaking the agar plates with the test organism, the disks were imbedded in the surface of the agar. In this way it was possible to demonstrate activity of a limited nature by most of the compounds tested.

While nearly all of the compounds displayed at least slight activity, only two could be considered significant. These were 1-hydroxy-4-dimethyl*aminoanthraquinone* and 6,9-dihydroxybenzo[g]-quinoline-5,10-dione. Neither, however, was as effective as tetracycline under the conditions employed.

EXPERIMENTAL

Syntheses

2-Hydroxybenzo[g]-quinoline-5,10-dione-4-carboxylic Acid.—This compound was synthesized according to the method of Etienne and Staehelin (4, 5), starting with 3-amino-2-naphthoic acid. As reported by these authors, decarboxylation occurred upon heating, and the 2-hydroxybenzo[g]-quinoline-5,10-dione sublimed, observed m.p. 341–343° (uncorrected). Reported m.p. 349–351° (5). If heated to 230–240° at 0.3 mm. Hg pressure, the acid sublimed without decomposition.

1-Hydroxy-4-dimethylaminoanthraquinone.—The acid sulfate of *p*-aminophenol was prepared by dissolving the phenol in an excess of 10% sulfuric acid and precipitating the salt by the addition of ethanol. After recrystallization from absolute ethanol, the melting point was found to be 308–310° (uncorrected).

A mixture of 7.0 Gm. (0.034 mole) of the salt, 11.3 Gm. (0.076 mole) phthalic anhydride, and 1.5

[1] The authors are indebted to Dr. Leon LeBeau, Department of Microbiology, College of Medicine, University of Illinois, and to Dr. Carmen Mascoli, Department of Microbiology, West Virginia University, for their assistance and advice in the testing procedures.

Gm. boric acid with 30.0 ml. concentrated sulfuric acid was heated slowly in an oil bath to a temperature of 160°, and maintained at this temperature for 8 hours. At the end of this time, the melt was cooled and poured on about 200 Gm. chipped ice with constant stirring. The reaction mixture was then carefully neutralized to approximately pH 7 by the addition of sodium bicarbonate. The mixture was evaporated to dryness, the residue powdered and extracted with chloroform using a Soxhlet extractor. The chloroform extract was evaporated to dryness and the crude product (7.5 Gm. = 93%) purified by recrystallization from 95% ethanol, followed by sublimation at 3 mm. Hg, and a bath temperature of 140°. The bright red needles darkened slightly at 168–170° and melted at 212–213° (uncorrected). Reported m.p., 215° (14).

A solution of 200 mg. (0.0084 mole) of 1-hydroxy-4-aminoanthraquinone in 10 ml. absolute methanol was treated with 5 Gm. (0.035 mole) methyl iodide and 0.1 Gm. anhydrous potassium carbonate. After refluxing on a steam bath for 6 hours, the solvent and excess methyl iodide were allowed to evaporate.

The dry residue was purified by sublimation under reduced pressure at 160–180°. Overall yield 155 mg. (69.7%) of orange needles, m.p. 204–205° (uncorrected).

Anal.—Calcd. for $C_{16}H_{13}NO_3$: C, 71.90; H, 4.90; N, 5.24. Found: C, 71.82; H, 4.93; N, 5.19.

Bis-(4-chlorophenyl) Quinolinate.—A mixture of 2.1 Gm. (0.013 mole) quinolinic acid and 6.4 Gm. (0.050 mole) *p*-chlorophenol was treated with 13.4 Gm. phosphoryl chloride, and the mixture refluxed 6 hours. After cooling, the reaction mixture was poured into ice water and the excess phosphoryl chloride allowed to decompose. The reddish-brown oily product was separated from the aqueous layer and treated with solid sodium carbonate. The resulting oily green material was dissolved in chloroform, the solution dried over anhydrous sodium sulfate, and the chloroform evaporated. The still-oily residue was dissolved in a small amount of boiling methanol and filtered. Water was added dropwise until the solution began to cloud. The mixture was agan heated to boiling, filtered, and the filtrate cooled in an ice bath. The resulting dark green crystals were removed by suction filtration and dried. Repeated recrystallization from methanol and water gave 2.1 Gm. dark green needles, m.p. 121–122° dec. (uncorrected).

Anal.—Calcd. for $C_{19}H_{11}NO_4Cl_2$: C, 58.78; H, 2.86; N, 3.61; Cl, 18.27. Found: C, 58.65; H, 2.91; N, 3.58; Cl, 18.20; sapon. equiv., 192.

6,9-Dihydroxybenzo[g]-quinoline-5,10-dione.—A mixture of 5.0 Gm. (0.030 mole) quinolinic acid and 33.5 Gm. phosphoryl chloride was heated under reflux for 45 minutes. At the end of this time, 1.5 Gm. (0.012 mole) hydroquinone monomethyl ether and 3.0 Gm. phosphorus pentoxide were added, and the mixture again refluxed for 3 hours. The cooled reaction mixture was poured on crushed ice to decompose the phosphorus pentoxide and phosphoryl chloride. The dark brown precipitate was removed by suction filtration and recrystallized from absolute methanol, yielding 1.7 Gm. (58.4%) dark purple leaflets, m.p. 308–310° (uncorrected).

Anal.—Calcd. for $C_{13}H_7NO_4$: C, 64.73; H, 2.92; N, 5.81. Found: C, 64.65; H, 2.95; N, 5.85.

Alternate Method.—A mixture of 8.0 Gm. (0.048 mole) quinolinic acid, 6.0 Gm. (0.043 mole) hydroquinone dimethyl ether, 10 Gm. phosphorus pentoxide and 42.0 Gm. phosphoryl chloride was refluxed for 5 hours. After the isolation and purification reported above, the yield was 7.4 Gm. (70.6%) of 6,9-dihydroxybenzo[g]-quinoline-5,10-dione, identical with that obtained above as established by analysis and mixed melting point.

6 - Hydroxy - 9 - chlorobenzo[g]quinoline - 5,10 - dione.—To a mixture of 42.0 Gm. phosphoryl chloride and 10 Gm. phosphorus pentoxide were added 5.0 Gm. (0.030 mole) quinolinic acid and 3.5 Gm. (0.027 mole) *p*-chlorophenol. After refluxing for 6 hours, the mixture was cooled and poured onto chipped ice. The dark orange precipitate was removed by filtration. Recrystallized from absolute methanol, the orange crystals melted at 318–320° (uncorrected). The yield was 6.8 Gm. (70.9%).

Anal.—Calcd. for $C_{13}H_6NO_3Cl$: C, 60.13; H, 2.33; N, 5.40; Cl, 13.66. Found: C, 60.02; H, 2.38; N, 5.45; Cl, 13.75.

6,9 - Dichlorobenzo[g]quinoline - 5,10 - dione.—A mixture of 8.0 Gm. (0.048 mole) quinolinic acid, 5.0 Gm. (0.034 mole) *p*-dichlorobenzene, 10.0 Gm. phosphorus pentoxide and 50.0 Gm. phosphoryl chloride was refluxed for 6 hours, cooled, then poured on chipped ice.

The reddish-brown tar-like mass was dissolved in acetone and reprecipitated by the addition of water. The resulting brown powder was recrystallized twice from 95% ethanol to give fine dark yellow crystals (4.6 Gm. = 48.4%), m.p. 316–317° (uncorrected).

Anal.—Calcd. for $C_{13}H_5NO_2Cl_2$: C, 56.14; H, 1.81; N, 5.04; Cl, 25.50. Found: C, 55.80; H, 1.85; N, 4.95; Cl, 25.12.

Bacteriological Screening

A weighed sample of each of the compounds was dissolved in absolute methanol, and the volume adjusted so that the final solution contained 0.300 mg./ml. Filter paper disks were impregnated by placing them on a glass plate and allowing 0.2 ml. of a solution of one of the compounds to flow slowly onto the surface of the disk. The solvent was then allowed to evaporate, leaving each of the disks impregnated with 60 mcg. of one of the compounds under test. For comparison of activity, disks containing 40 mcg. of tetracycline were used.

The impregnated disks were placed on tryptose-glucose-agar plates inoculated with one of the following organisms: β-hemolytic *Strep. pyogenes*, *Proteus vulgaris*, *Escherichia coli*, *Staph. aureus*, *Salmonella enteritidis*, *Bacillus cereus* var. *terminalis*. The disks were gently pressed directly into the surface of the agar with the aid of a sterile glass rod. After covering, the cultures were incubated at 37° for 24 hours, then examined for inhibition of growth of the organisms.

The results obtained are shown in Table I.

SUMMARY

The following hitherto unreported compounds were synthesized and screened for bacteriostatic

TABLE I.—INHIBITION OF GROWTH OF TEST ORGANISMS[a]

Test Compound	*Strep. pyogenes*, β-hemolytic	*P. vulgaris*	*E. coli*	*Staph. aureus*	*Salmonella enteritidis*	*B. cereus* var. *terminalis*
1-Hydroxy-4-aminoanthraquinone	−	−	−	−	±	++
1-Hydroxy-4-dimethylaminoanthraquinone	−	+	+	−	−	−
6,9-Dihydroxybenzo[g]quinoline-5,10-dione	++	−	±	−	±	−
6-Hydroxy-9-chlorobenzo[g]quinoline-5,10-dione	−	−	−	−	+	−
6,9-Dichlorobenzo[g]quinoline-5,10-dione	−	−	−	−	−	−
Bis-(4-chlorophenyl)quinolinate	−	−	−	−	−	−
2-Hydroxybenzo[g]quinoline-5,10-dione-4-carboxylic acid	+	±	−	−	−	−
Tetracycline (40 mcg.)	+	−	++	++	−	+

[a] Radii of zones of inhibition represented by different symbols are: (−) No inhibition. (±) Less than 2 mm. (+) 2–10 mm. (++) Greater than 10 mm.

activity: 6,9-dihydroxybenzo[g]quinoline-5,10-dione(I), 6-hydroxy-9-chlorobenzo[g]quinoline-5,10-dione(II), 6,9-dichlorobenzo[g]quinoline-5,10-dione(III), bis-(4-chlorophenyl) quinolinate (IV), 1-hydroxy-4-dimethylaminoanthraquinone(V).

In addition, two previously reported compounds were prepared and tested: 2-hydroxybenzo[g] quinoline-5,10-dione-4-carboxylic acid(VI) and 1-hydroxy-4-aminoanthraquinone(VII).

While most of the compounds tested showed some degree of inhibition to the growth of some organisms, none were found to approach tetracycline in effectiveness.

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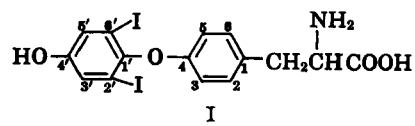
Thyroxine Analogs IX

4′-Deoxy-4′-β-L-alanyl-3,5-diiodo-L-thyronine and Related Stereoisomers as Thyroxine Antagonists

By EUGENE C. JORGENSEN and RICHARD CAVESTRI

On the basis of structural considerations and the activity of 2′,6′-diiodothyronine (I), 4′-deoxy-4′-β-L-alanyl-3,5-diiodo-L-thyronine (VIIb)[1] and a mixture of related stereoisomers have been prepared and tested for activity as thyroxine antagonists in the rat antigoiter assay. The L,L-isomer (VIIb) proved moderately effective as an antagonist at a molar ratio to L-thyroxine of 100:1. The mixed DL,DL-isomers (VIIa) as well as the L,L-isomer (VIIb) were ineffective when tested at a molar ratio to L-thyroxine of 200:1.

STUDIES directed at the preparation of peripheral antagonists to the thyroid hormones have had two principal objectives: (*a*) the development of an agent capable of rapidly reducing the undesirable effects due to high levels of circulating hormone in the various forms of hyperthyroidism, and (*b*) the gaining of a better understanding of the nature of interactions between the thyroid hormones and their biological receptor sites (1). 2′,6′-Diiodo-DL-thyronine (I) was the first compound containing the intact thyronine nucleus characteristic of thyroxine which was shown to possess thyroxine antagonistic properties. Among a number of halogenated thyronines prepared by Niemann and McCasland (2) and tested by Cortell (3),



I

only the 2′,6′-diiodo analog (I) showed thyroxine

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This work was supported by Research Grant A 4223, from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service, Bethesda, Md.
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1-Hydroxy-4-dimethylaminoanthraquinone	−	+	+	−	−	−
6,9-Dihydroxybenzo[g]quinoline-5,10-dione	++	−	±	−	±	−
6-Hydroxy-9-chlorobenzo[g]quinoline-5,10-dione	−	−	−	−	+	−
6,9-Dichlorobenzo[g]quinoline-5,10-dione	−	−	−	−	−	−
Bis-(4-chlorophenyl)quinolinate	−	−	−	−	−	−
2-Hydroxybenzo[g]quinoline-5,10-dione-4-carboxylic acid	+	±	−	−	−	−
Tetracycline (40 mcg.)	+	−	++	++	−	+

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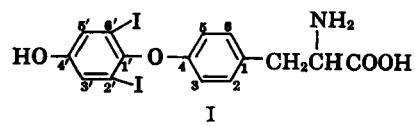

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antagonistic activity when tested together with thyroxine at a molar ratio of 150:1 in the rat antigoiter assay. This compound has been extensively studied since. Antagonistic activity in the rat antigoiter assay has been confirmed (4) as well as the absence of detectable thyroxine-like activity in the rat and in man (5). Weak activity has been shown by the DL-isomer in antagonizing the thyroxine-induced elevation of oxygen consumption in the mouse (6).

With the exception of 2′,6′-diiodothyronine, all thyronine derivatives thus far studied have required at least one substituent in the 3 or 5 positions of the alanine-bearing ring for the presence of either thyroxine-like or antagonistic activity. For example, thyronine and its 3′-iodo- and 3′,5′-diiodo-derivatives have been shown to possess neither thyroxine-like (7–10) nor thyroxine-antagonistic (11, 12) properties. Thyronine derivatives with a single iodine atom in the alanine-bearing ring, *e.g.*, 3-iodo-, 3,3′-diiodo-, and 3,3′,5′-triiodo-DL-thyronines, have been shown to act as thyroxine antagonists by a variety of biological tests (12). 3,3′-Diiodothyronine (L-isomer (10), DL-isomer (13)), and 3,3′,5′-triiodo-DL-thyronine (14) have also been reported to show weak thyroxine-like activity. Maximal thyroxine-like activity in mammals has been invariably associated with the 3,5-diiodotyrosinyl grouping present in the natural hormones, L-thyroxine and 3,3′,5-triiodo-L-thyronine (7, 15). These consistent structural requirements for 3 or 3,5 substitution in the thyronine nucleus have led to the hypothesis (16) that the ring bearing these substituents may be responsible for primary binding to the biological receptor. Appropriate activating groups in the 3 or 5 positions (such as halogen or methyl (17)) may then function by contributing to the binding forces between the ring bearing these groups and the biological receptor. Bulky 3,5 substituents may also serve by steric effects to orient the two aromatic rings of the thyroid hormones and their analogs, so that the planes of the rings are perpendicular to each other. The suitably oriented phenolic ring with its hydroxyl group conjugated 1,4 to the ether oxygen may then comprise the functional unit of the thyromimetic molecule (16).

A peripheral antagonist may be visualized as one capable of binding to the hormonal receptor site but considerably less effective than the natural hormone in its functional role. Since in all other compounds tested 3 substitution is a requirement for minimal thyroxine-like activity, it seems possible that the iodine-bearing ring of 2′,6′-diiodothyronine may be the portion of the molecule fulfilling the binding role of the 3,5-diiodophenyl ring of thyroxine. Interaction of the 2′,6′-diiodo analog (I) with a hypothetical receptor structure (16) may be pictured as in Fig. 1. If so bound, the alanyl side chain of 2′,6′-diiodothyronine would occupy a position in space normally filled by the 4′-hydroxyl group of thyroxine. Phenyl ethers of 3,5-diiodotyrosine with a methyl group blocking the 4′ position against potential metabolic hydroxylation have shown thyroxine antagonistic properties in the rat antigoiter assay (16, 18). If the normal binding and functional roles of the alanine-bearing and phenolic rings are reversed with 2′,6′-diiodothyronine, the functional requirement for a 4′-hydroxyl group is not met and the alanyl residue acts as a 4′ blocking group. The molecule contains features which would be expected to facilitate transport to the biological receptor site—the alanyl group and a weakly dissociated phenolic group. It also possesses the diiodophenoxy structure for binding to the receptor, but if so bound, it lacks the structural characteristics at the "functional receptor" (Fig. 1) necessary for the functional role of a thyroxine-like compound—a hydroxyl group in 1,4 conjugation to the ether oxygen.

Because of the potential ability for an amino acid side chain to form ionic and hydrogen bonds with functional groups which might occupy positions peripheral to the receptor area, it was considered that the alanyl group could serve both as a blocking group and one providing a more firmly bound drug-receptor complex. To test this concept, a thyroxine analog possessing the 3,5-diiodotyrosinyl inner ring and a 4′-alanyl blocking group in the outer ring was prepared. The L-alanyl side chain in the inner ring was considered the most desirable for transport and binding since L-thyroxine and related

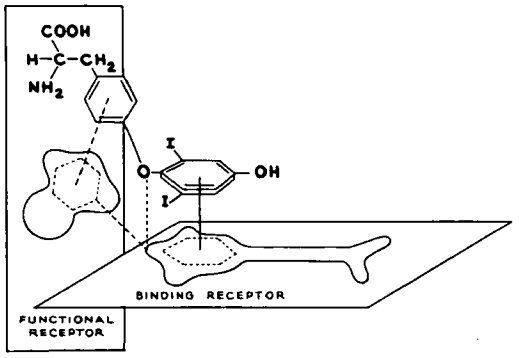


Fig. 1.—Schematic representation of 2′,6′-diiodothyronine interacting with a hypothetical thyroid hormone receptor.

L analogs display strong hormonal activity, while the corresponding D isomers possess relatively little (7). However, the more desirable configuration for the 4′-alanyl group (if such exists) could not be deduced, since activity has been reported only for the DL isomer of 2′,6′-diiodothyronine (3, 4, 6). Therefore, both the DL,DL compound (VIIa), comprising a mixture of all optical forms, and one of its enantiomers, the L,L compound (VIIb), were prepared.

The analogs were synthesized by the Meltzer (19) modification of the method of Chalmers, *et al.* (20). The appropriate DL or L isomer of N-acetyl 3,5-dinitrotyrosine ethyl ester was allowed to react with methanesulfonyl chloride and pyridine to form the N-acetyl 3,5-dinitro-4-pyridinium phenylalanine ethyl ester methanesulfonate (IIa,b). This was not isolated but was allowed to react with either the DL or L isomer of N-acetyl tyrosine ethyl ester (IIIa,b) to form the substituted dinitrodiphenyl ethers (IVa,b). These were converted to the diamines by hydrogenation and were bis-diazotized under anhydrous conditions without isolation and converted to the diiodo compounds (Va,b). Mild alkaline hydrolysis afforded the di-N-acetyl derivative (VI), while acidic conditions yielded the desired bis-amino acids (VIIa,b).

EXPERIMENTAL

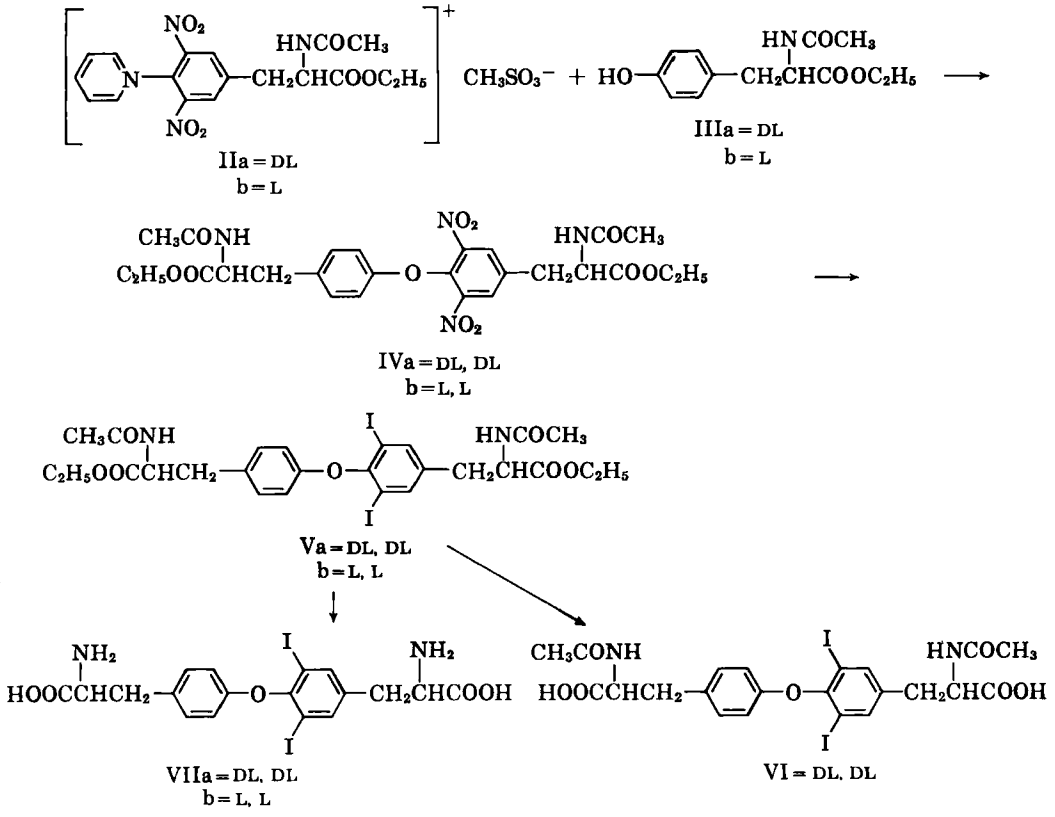
Synthetic

All melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were measured with a Rudolph polarimeter. Microanalyses were carried out by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley.

N-Acetyl 3,5-Dinitro-4-(N-acetyl 4′-DL-phenylalanyloxy ethyl ester)-DL-phenylalanine Ethyl Ester (IVa).—To a solution of N-acetyl 3,5-dinitro-DL-tyrosine ethyl ester (21) (17.0 Gm., 0.05 mole) in dry pyridine (100 ml.), methanesulfonyl chloride (6.3 Gm., 0.055 mole) was added, and the mixture heated under reflux for 2 minutes. N-Acetyl DL-tyrosine ethyl ester (2) (IIIa, 15.9 Gm., 0.063 mole) was added and the solution heated under reflux for 15 minutes. The reaction mixture was poured into cold water (200 ml.) and extracted with benzene and chloroform. The combined extracts were washed successively with 2 *N* hydrochloric acid, water, 0.3 *N* sodium hydroxide, and water. The residue obtained following removal of the solvents under reduced pressure was crystallized from aqueous ethanol yielding 13.7 Gm. (46%), m.p. 171–172°.

Anal.—Calcd. for $C_{26}H_{30}N_4O_{11}$; C, 54.35; H, 5.25. Found: C, 54.30; H, 5.41.

N-Acetyl 3,5-Dinitro-4-(N-acetyl 4′-L-phenylalanyloxy ethyl ester)-L-phenylalanine Ethyl Ester (IVb).—The condensation of N-acetyl 3,5-dinitro-L-tyrosine ethyl ester (20) and N-acetyl L-tyrosine ethyl ester (6) (IIIb) was carried out as described above, except that reflux time was increased from 15



to 30 minutes. Crystallization from 50% ethanol yielded 16.5 Gm. (58%), m.p. 163–164°. $[\alpha]_D^{23}$ −8.0° (*c* 2.00, $CHCl_3$).

Anal.—Calcd. for $C_{26}H_{30}N_4O_{11}$; C, 54.35; H, 5.25. Found: C, 54.52; H, 5.33.

N-Acetyl 3,5-Diiodo-4-(N-acetyl 4′-DL-phenylalanyloxy ethyl ester)-DL-phenylalanine Ethyl Ester (Va).—The dinitro compound (IVa, 10.7 Gm., 0.019 mole) dissolved in acetic acid (200 ml.) was shaken for 45 minutes in the presence of palladium-on-charcoal (10%, 2.0 Gm.) and hydrogen (45 p.s.i. initial pressure). Concentrated sulfuric acid (40 ml.) was added with cooling. The catalyst was removed by filtration through Celite, and the solution of the diamine was added from a pressure-equalized dropping funnel under nitrogen during 1 hour to a well-stirred mixture of nitrosylsulfuric acid maintained at −5°. The nitrosylsulfuric acid was prepared from sodium nitrite (5.52 Gm., 0.08 mole), added in small portions to concentrated sulfuric acid (60 ml.) at 60–70°, then diluted when cool with acetic acid (60 ml.). After the addition was complete, the dark solution of the bis-diazonium compound was stirred for an additional hour at −5°, then poured rapidly into a well-stirred mixture at 5° of iodine (21.4 Gm., 0.084 mole), sodium iodide (30.0 Gm., 0.2 mole), urea (3.12 Gm., 0.052 mole), water (300 ml.), and chloroform (300 ml.). After stirring for 1 hour below 15°, the reaction mixture was allowed to reach room temperature and stirring was continued for an additional hour. The chloroform phase was removed, the aqueous layer was extracted with chloroform, and the combined chloroform extracts were washed successively with water, 10% aqueous sodium bisulfite, water, 1 *M* sodium bicarbonate, and water. The chloroform extract was dried over anhydrous sodium sulfate, and the chloroform removed at reduced pressure. The residue was taken up in acetone, and the oil which precipitated upon addition of an equal volume of ligroin (b.p. 35–60°) crystallized after standing for about 1 month at 0° yielding 4.2 Gm. (35%), m.p. 140–143°.

Anal.—Calcd. for $C_{26}H_{30}I_2N_2O_7$; C, 42.41; H, 4.11. Found: C, 42.89; H, 4.35.

N-Acetyl 3,5-Diiodo-4-(N-acetyl 4′-L-phenylalanyloxy ethyl ester)-L-phenylalanine Ethyl Ester (Vb).—The L,L-dinitro compound (IVb, 3.3 Gm., 0.0058 mole) was treated in the same way as above. The oily residue obtained crystallized readily on scratching and was recrystallized from ethanol yielding 2.4 Gm. (57%), m.p. 197–198°. $[\alpha]_D^{23}$ −6.3° (c 2.00, $CHCl_3$).

Anal.—Calcd. for $C_{26}H_{30}I_2N_2O_7$; C, 42.41; H, 4.11. Found: C, 42.67; H, 4.09.

N-Acetyl 3,5-Diiodo-4-(N-acetyl 4′-DL-phenylalanyloxy)-DL-phenylalanine (VI).—The bis-N-acetyl amino acid ethyl ester (Va, 1.11 Gm., 1.5 mmoles) was dissolved in absolute ethanol (30 ml.), sodium hydroxide (1.12 Gm., 30 mmoles) was added, and the mixture was stirred at room temperature for 2 hours. The solution was diluted with an equal volume of hot water, acidified to pH 3 with 2 *N* hydrochloric acid and allowed to crystallize overnight at 0°. Recrystallization from aqueous acetone yielded 0.27 Gm. (38%), m.p. 143–145°.

Anal.—Calcd. for $C_{22}H_{22}I_2N_2O_7$; C, 38.85; H, 3.26. Found: C, 38.6; H, 3.4.

3,5-Diiodo-4-(4′-DL-phenylalanyloxy)-DL-phenylalanine (VIIa).—The bis-N-acetyl amino acid ethyl ester (Va, 1.0 Gm.) was heated under reflux for 12 hours in glacial acetic acid (10 ml.) and concentrated hydrochloric acid (10 ml.). The mixture was treated with decolorizing carbon and taken to dryness under reduced pressure. The cream-colored residue was dissolved in 15 ml. of hot water with the aid of a few drops of concentrated hydrochloric acid. The hot solution was adjusted to pH 5 with a saturated solution of sodium acetate. After three such isoelectric precipitations, the white amino acid weighed 0.337 Gm. (37%), m.p. approximately 350° dec.

Anal.—Calcd. for $C_{18}H_{18}I_2N_2O_5 \cdot H_2O$; C, 35.21; H, 3.48. Found: C, 35.41; H, 3.57. On a separate sample dried at 65° (2 mm.)—Calcd. for $C_{18}H_{18}I_2N_2O_5$; I, 42.53. Found: I, 42.48.

3,5-Diiodo-4-(4′-L-phenylalanyloxy)-L-phenylalanine Dihydrochloride (VIIb).—The bis-N-acetyl amino acid ethyl ester (Vb, 1.0 Gm.) was heated under reflux for 12 hours in glacial acetic acid (10 ml.) and concentrated hydrochloric acid (10 ml.). The clear solution was taken to dryness at reduced pressure. The solid residue was dissolved by heating in boiling water (15 ml.) and concentrated hydrochloric acid (1 ml.). Cooling yielded the crystalline dihydrochloride, 0.832 Gm. (93%), m.p. 293–295° dec. $[\alpha]_D^{23}$ −1.6° (c 2.00, 1 *N* sodium hydroxide-ethanol (1:2)).

Anal.—Calcd. for $C_{18}H_{18}I_2N_2O_5 \cdot 2HCl$; C, 32.34; H, 2.99; I, 37.81. Found: C, 32.5; H, 2.9; I, 37.7.

Biological

The rat antigoiter assay was based on that of Dempsey and Astwood (22) and Cortell (3), in which thyromimetic activity was estimated by determining the amount of the test compound required to prevent the increase in thyroid weight brought about by the goitrogen, thiouracil. Thyroxine-antagonistic activity was tested by the ability of the test compound to reverse the antigoiter effect of thyroxine when administered concomitantly in thiouracil-fed rats. The assay was carried out as previously described (16). Specific assay conditions and results are collected in Table I.

DISCUSSION

At a molar ratio to L-thyroxine of 100:1, the L,L-isomer (VIIb) produced a 46.5% reversal[2] of the goiter preventing effect of thyroxine (Table I, assay 1). When the data was examined by the student's "t" test, the probability that the thyroid weight produced by thiouracil, thyroxine, and VIIb (14.9 ± 3.0 mg./100 Gm.) was the same as that produced by thiouracil and thyroxine alone (10.3 ± 4.0 mg./100 Gm.) was $P < 0.10$. Thus a moderate degree of antagonism of the antigoitrogenic effect of thyroxine was indicated. This is the same order of effectiveness reported[3] for 2′,6′-DL-diiodothyronine, where a 63% reversal of the effect of DL-thyroxine was observed at a molar ratio of 82:1; a constant reversal of between 43–48% was observed in a duplicate assay at molar ratios of 82:1,

[2] For the method of calculation see Reference 18.

[3] Values for per cent reversal and molar ratios were calculated from the data presented in Reference 4.

TABLE I.—RAT ANTIGOITER ASSAY OF 4′-DEOXY-4′-ALANYL-3,5-DIIODOTHYRONINES

Assay No.	Food	Compound Injected[a]	Daily Dose per 100 Gm., mcg.	Molar Ratio	Thyroid Wt. per 100 Gm. mg.	± s.d.
1[b]	Untreated				6.6	0.8
	TU[c]				20.2	3.9
	TU	Thyroxine	2.0	0.67	14.4	3.5
	TU	Thyroxine	3.0	1.0	10.3	4.0
	TU	Thyroxine	4.5	1.5	6.1	3.0
	TU	Thyroxine plus L,L-isomer (VIIb)	3.0 225	1.0 100	14.9[d]	3.0
2[b]	Untreated				8.2	1.6
	TU[c]				29.5	4.5
	TU	Thyroxine	2.0	0.67	18.2	5.2
	TU	Thyroxine	3.0	1.0	10.7	3.6
	TU	Thyroxine	4.5	1.5	9.1	3.9
	TU	Thyroxine plus L,L-isomer (VIIb)	3.0 450	1.0 200	12.7[e]	3.6
	TU	Thyroxine plus DL,DL-isomers (VIIa)	3.0 400	1.0 200	10.6[f]	2.6

[a] Thyroxine was used as sodium L-thyroxine pentahydrate, kindly provided by Dr. James Kerwin, Smith Kline and French Laboratories. [b] Six rats at each control and dose level. [c] Thiouracil 0.3%. [d] Reversal of thyroxine effect, 46.5% ($P < 0.10$). [e] Reversal of thyroxine effect, 11%; not significant. [f] No reversal of thyroxine effect.

164:1, and 328:1. This difficulty in producing a greater response with increased dose or duplicating the level of significance found earlier was observed in the second assay shown in Table I. At a molar ratio to L-thyroxine of 200:1, the L,L-isomer (VIIb) produced an 11% reversal of thyroxine's antigoitrogenic effect, a value which was not significantly different from the thyroxine-control value. The mixture of DL,DL-isomers (VIIa) had no effect at a molar ratio to L-thyroxine of 200:1 in the same assay.

The results with the L,L-isomer (VIIb) are indicative of a low order of thyroxine antagonistic activity. However, the inability to obtain reproducible and significant results in the rat antigoiter assay makes it undesirable to use this data in support of the proposals put forth regarding the nature of the antagonistic activity demonstrated by 2′,6′-diiodothyronine without further evaluation in other test systems. Since such evaluation will not be possible in the near future, the rationale for the preparation of these compounds and the results obtained to date are presented now.

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Prolongation of Chlorzoxazone Plasma Levels by Zoxazolamine

By JOHN W. POOLE and JOSEPH F. GARDOCKI

The plasma levels of chlorzoxazone were determined in humans, dogs, and mice. It was shown that the co-administration of zoxazolamine and chlorzoxazone prolonged the plasma concentrations of the latter substance in mice and dogs. An interaction between the compounds under consideration, which resulted in the formation of an insoluble complex, was demonstrated by means of a solubility study. Evidence was presented to show that the prolongation of chlorzoxazone plasma levels was probably a result of a change in metabolism of this compound in the presence of the additive, zoxazolamine. It is suggested that the molecular complex formed between chlorzoxazone and zoxazolamine is directly responsible for the proposed alteration in metabolism of chlorzoxazone and the prolonged plasma levels of this compound which results when the combination of drugs is administered.

CHLORZOXAZONE (5-chlorobenzoxazolinone) is a centrally acting muscle relaxant which has been found useful in the treatment of a number of spastic diseases. The physiological disposition and metabolic fate of this compound have been reported previously by Conney and Burns (1). The present investigation is concerned with the absorption and biological life of this substance in several animal species after the administration of the drug alone and in combination with a potentiating agent.

Specifically, the animal species employed in this investigation were mice, dogs, and humans, and the potentiating agent utilized was zoxazolamine (2-amino-5-chlorobenzoxazole). The potentiating effect of the additive was demonstrated by a prolongation of chlorzoxazone plasma levels when the combination of drugs was administered.

The basis for investigating the chlorzoxazone-zoxazolamine combination was the hypothesis developed by Brodie, Hogben, Schanker, and associates (2–4) to explain the rate and extent of absorption of acidic and basic drugs from the gastrointestinal tract. It was thought that administration of chlorzoxazone (the acidic drug) and zoxazolamine (the basic compound) would demonstrate a more prolonged muscle relaxant activity than either of the compounds administered singly since the weakly acidic drug would be absorbed more readily in the acidic portion of the gastrointestinal tract, and the weakly basic drug in the more basic segments of the tract. However, it was noted that the chlorzoxazone plasma levels were appreciably prolonged when the combination of drugs was administered when compared to the levels observed after administration of chlorzoxazone alone.

The purpose of this study was to characterize the absorption pattern of chlorzoxazone in various animal species with the possibility of utilizing this information in the development of more efficient dosage forms, and to investigate the mechanism responsible for the prolongation of chlorzoxazone plasma levels noted when this compound is administered in conjunction with zoxazolamine.

EXPERIMENTAL

The estimation of chlorzoxazone levels in the biological samples was made by the method described by Conney, *et al.* (5). This procedure involved extraction of acidified biological material with an organic solvent consisting of 1.5% isoamyl alcohol in petroleum ether. The drug was then extracted from the organic phase into a 0.5 *N* sodium hydroxide solution and measured spectrophotometrically at 287 mμ where the compound exhibits an absorption maximum.

The method employed for the determination of zoxazolamine was similar to that described for chlorzoxazone. However, the drug was extracted from alkalinized biological material into the organic solvent and re-extracted into a 3 *N* hydrochloric acid solution. Measurements were made spectrophotometrically at 278 mμ.

The experimental procedure utilized to demonstrate the interaction between chlorzoxazone and zoxazolamine in distilled water and mouse plasma was similar to that reported by Higuchi and Lach (6). The analytical procedures employed were similar to those described for the biological samples.

The spectrophotometric measurements were made on a Cary model 14 recording spectrophotometer.

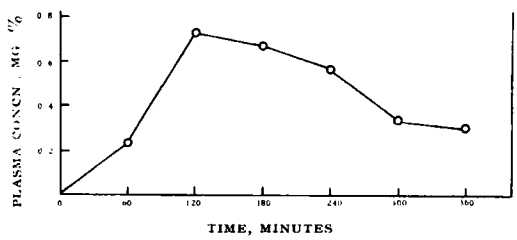

Fig. 1.—Mean plasma levels of chlorzoxazone in humans after oral administration of 0.50 Gm. of drug.

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RESULTS AND DISCUSSION

Ten humans received 0.50 Gm. of chlorzoxazone orally in tablet form, and the plasma levels were measured at various time intervals. Blood samples were obtained from five of the subjects at 0, 60, 180, and 300 minutes and from the remaining five subjects at 0, 120, 240, and 360 minutes. Figure 1 presents the mean plasma levels observed in this study. Peak plasma levels were attained 120–180 minutes after administration and the biological half-life was determined to be about 170 minutes. These data are consistent with the duration of action noted with this compound in clinical studies. In addition, the results noted in this investigation are in general agreement with the observations previously reported by Conney and Burns (1).

The mean plasma levels observed in dogs after oral administration of 30 mg./Kg. and 60 mg./Kg. chlorzoxazone are shown in Fig. 2. Six dogs were used at each dose level, and the drug was administered in capsule form. The biological half-life in this species was determined to be about 120 minutes with a peak level observed between 90–180 minutes. Figure 2 also shows the mean plasma levels of chlorzoxazone observed after administration of 60 mg./Kg. chlorzoxazone in conjunction with 60 mg./Kg. zoxazolamine. Four dogs were employed on this dosage schedule. Although the peak concentration of chlorzoxazone did not differ significantly from that obtained after administration of chlorzoxazone alone, there was a definite prolongation of the plasma concentration. From the limited data presented, it appears that the half-life of chlorzoxazone is prolonged to about 300 minutes when this compound is administered with an equivalent dose of zoxazolamine.

The plasma levels of chlorzoxazone determined after administration of a suspension of the drug to mice at levels of 100 mg./Kg. and 200 mg./Kg. are shown in Fig. 3. The chlorzoxazone determinations were made on a pooled blood sample from about 20 mice. The peak chlorzoxazone levels in this species were noted at 5 minutes with a half-life of only 20 minutes. The short half-life observed in this species is consistent with the data reported by Conney and Burns (1) which showed that liver homogenates prepared from mouse or guinea pig tissue metabolized chlorzoxazone three to four times as fast as a corresponding preparation using rat or rabbit tissue. The chlorzoxazone plasma levels attained in mice after oral administration of 100 mg./Kg. each of chlorzoxazone and zoxazolamine are also shown in Fig. 3. The plasma concentrations reported for the combination are the means of three separate sets of pooled blood samples. The results were similar to those observed with dogs since there was a significant prolongation of the chlorzoxazone plasma concentrations when the combination of drugs was given. The biological half-life of chlorzoxazone in this instance was about 80 minutes or four times longer than that noted when chlorzoxazone was administered singly.

Several of the possible mechanisms which could account for the prolonged chlorzoxazone plasma concentrations demonstrated after administration of the combination of drugs are (*a*) conversion of zoxazolamine to chlorzoxazone in the biological system, (*b*) prolonged dissolution, and therefore prolonged absorption due to the formation of a poorly soluble interaction product of chlorzoxazone and zoxazolamine, (*c*) an increase in absorption from the gastrointestinal tract of chlorzoxazone in the presence of zoxazolamine, (*d*) lower chlorzoxazone tissue levels (or more rapid removal of this drug from the tissues when zoxazolamine is also in the system), and (*e*) an alteration in the metabolism of chlorzoxazone when both compounds are present.

It is known that 2-aminobenzoxazoles are converted *in vitro* to 2-benzoxazolinones in dilute mineral acid. However, the *in vivo* conversion of zoxazolamine to chlorzoxazone as the mechanism responsible for the prolongation of chlorzoxazone plasma levels was readily eliminated when no detectable levels of chlorzoxazone were found after administration of 60 mg./Kg. of zoxazolamine to dogs and 100 mg./Kg. of this agent to mice.

Although it is possible that prolonged dissolution and absorption occur when chlorzoxazone and

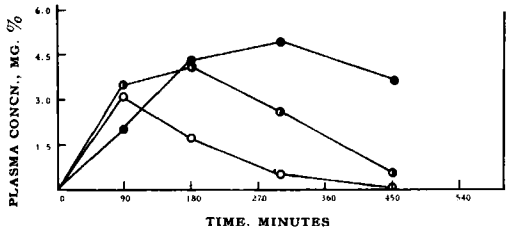


Fig. 2.—Mean plasma levels of chlorzoxazone in dogs after oral administration of 30 mg./Kg. (O) and 60 mg./Kg. (◑) of the drug alone and after administration of 60 mg./Kg. each of chlorzoxazone and zoxazolamine (●).

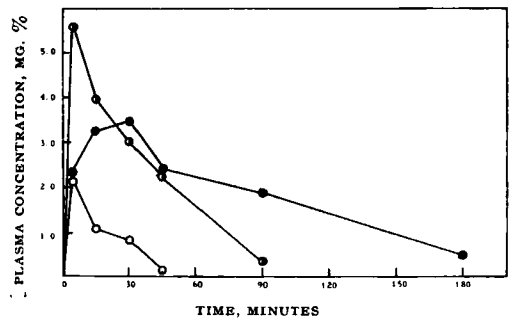


Fig. 3.—Plasma levels of chlorzoxazone in mice after oral administration of 100 mg./Kg. (O) and 200 mg./Kg. (◑) of the drug alone and after administration of 100 mg./Kg. each of chlorzoxazone and zoxazolamine (●).

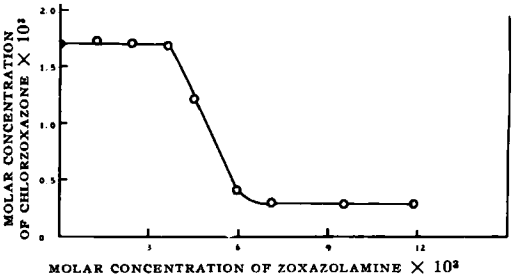


Fig. 4.—Phase diagram showing the effect of zoxazolamine on the apparent solubility of chlorzoxazone in water at 30°C.

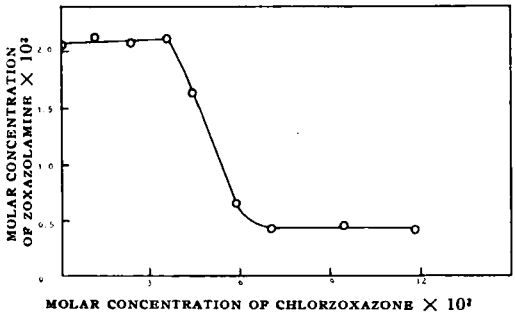


Fig. 5.—Phase diagram showing the effect of chlorzoxazone on the apparent solubility of zoxazolamine in water at 30°C.

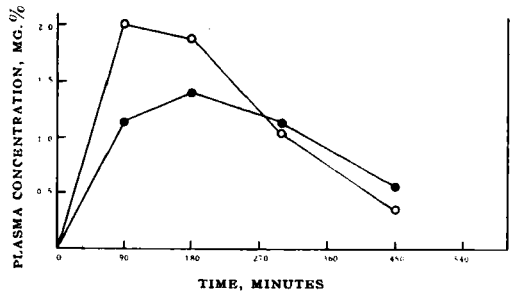
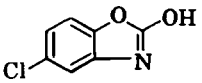


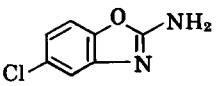
Fig. 6.—Mean plasma levels of zoxazolamine in dogs after oral administration of 60 mg./Kg. (O) of the drug alone and after administration of 60 mg./Kg. each of zoxazolamine and chlorzoxazone (●).

zoxazolamine are administered simultaneously (due to their interaction at the absorption site), this mechanism alone would not seem to explain adequately the large areas under the time-concentration curves observed for the drug combination.

The remaining possible mechanisms listed above also may be rationalized on the basis of an interaction between these muscle relaxant compounds. An examination of the chemical structure of the compounds under consideration shows that the structural requirements necessary for the formation of a molecular complex in aqueous solution similar to those described in previous communications (6–8) are present in these substances. The expected interaction was illustrated by means of a solubility study. The phase diagrams describing this interaction are



Chlorzoxazone



Zoxazolamine

shown in Figs. 4 and 5. Analysis of the phase diagrams and chemical analysis of the relatively insoluble complex formed showed the addition product to be composed of one molecule of chlorzoxazone to one of zoxazolamine.

It is unlikely that the complex demonstrated in aqueous solution would be stable in the acidic medium of the stomach. However, if the formation of a complex between these compounds was responsible for an increase in absorption of chlorzoxazone from the gastrointestinal tract, a corresponding increase in zoxazolamine plasma levels would also be observed. That this does not occur is demonstrated in Fig. 6 which shows the mean plasma levels of zoxazolamine after oral administration to dogs of 60 mg./Kg. zoxazolamine and 60 mg./Kg. zoxazolamine in combination with a like amount of chlorzoxazone. Similar results were noted with the plasma levels attained in mice after oral administration of 100 mg./Kg. zoxazolamine alone and in combination with 100 mg./Kg. chlorzoxazone. The early levels noted in both species were lower when the combination of drugs was administered than those observed when zoxazolamine was given alone. This may be a result of a lowering of the solubility or a slower dissolution rate of the zoxazolamine in the presence of chlorzoxazone in the gastrointestinal tract.

If an increase in gastrointestinal absorption is not

Table I.—Plasma Levels of Chlorzoxazone in Mice After Intraperitoneal Administration (mg. per cent)

	Time, Minutes					
	5	15	30	45	90	180
Chlorzoxazone, 100 mg./Kg.	6.80	3.66	0.73	0.81	0	0
Chlorzoxazone, 100 mg./Kg. Zoxazolamine, 100 mg./Kg.	6.22	3.25	3.96	2.31	1.65	0

Table II.—Brain and Plasma Levels of Chlorzoxazone in Mice After Oral Administration (mg. per cent)

		Time, Minutes				
		15	30	45	90	180
Chlorzoxazone alone	Brain	1.67	1.26	1.23	0	0
	Plasma	3.97	2.98	2.24	0.33	
Chlorzoxazone in combination	Brain	1.95	3.16	1.87	1.20	0
	Plasma	5.37	4.82	3.69	2.21	0.33

Table III.—Brain and Plasma Levels of Zoxazolamine in Mice After Oral Administration (mg. per cent)

		Time, Minutes				
		15	30	45	90	180
Zoxazolamine alone	Brain	2.05	1.24	0.99	0.35	0
	Plasma	0.69	0.36	0.15	0.06	0
Zoxazolamine in combination	Brain	0.73	0.60	0.60	0.20	0
	Plasma	0.54	0.36	0.32	0.22	0

responsible for the prolongation of the chlorzoxazone levels noted, then administration of the combination by other than the oral route should also result in a prolongation of chlorzoxazone plasma levels. Table I shows the chlorzoxazone plasma levels determined after intraperitoneal administration of 100 mg./Kg. chlorzoxazone alone and in combination with 100 mg./Kg. zoxazolamine. It is apparent from these results that this route of administration affects the chlorzoxazone levels in a manner similar to that observed after oral administration of the drug combination.

A comparison of Table I with Fig. 3 shows that at times greater than 5 minutes post administration the plasma levels resulting from the intraperitoneal route are similar to those observed when the same drug combination was administered orally to mice.

In order to obtain evidence for the *in vivo* formation of a complex between the compounds under consideration, a solubility study was conducted utilizing mouse plasma as the solvent and maintaining the temperature at 37.5°. Because of the limited number of experimental points available, it was not possible to analyze the phase diagram as was the case for the aqueous study described previously. However, evidence of complex formation in this system was obtained since (in each instance) the presence of one of the molecules in the system significantly decreased the solubility of the other in the biological fluid.

The effect of administration of the combination of drugs on the chlorzoxazone tissue levels was made by determining chlorzoxazone levels in the brains of mice. The dosage regimen utilized for this part of the study was 200 mg./Kg. chlorzoxazone alone and 200 mg./Kg. chlorzoxazone in combination with 50 mg./Kg. zoxazolamine. The brains of ten mice were pooled and homogenized with distilled water and an aliquot of the final preparation was used for the drug determination.

The results of this experiment, along with the chlorzoxazone plasma levels attained employing the same dosing schedule, are presented in Table II.

The data indicate that the brain levels of chlorzoxazone are proportional to the plasma levels of this drug, and that the prolongation of plasma concentrations is reflected in prolonged tissue concentrations. Therefore, the prolonged chlorzoxazone plasma levels noted in this study are probably not the result of a lowering or more rapid depletion of tissue chlorzoxazone in the presence of zoxazolamine.

The brain levels of zoxazolamine observed after oral administration of 50 mg./Kg. of this compound alone and in combination with 200 mg./Kg. chlorzoxazone are shown in Table III along with the plasma concentrations of zoxazolamine determined at these same dosage levels.

It is interesting to note that the brain levels of zoxazolamine are considerably lower after administration of the drug combination than those observed after administration of the zoxazolamine alone. However, there was no significant difference in the plasma levels noted after either dosage regimen was used. These results may be rationalized on the basis of complex formation between the muscle relaxant compounds. Since a large excess of chlorzoxazone molecules (in relation to zoxazolamine) is available in the system, the tissue distribution of this material would not be expected to be influenced significantly by the formation of the proposed complex. However, because of the relatively low concentration of zoxazolamine present, the distribution of this compound in the various biological compartments could be considerably altered by an interaction of the type suggested.

The data indicate that the prolongation of chlorzoxazone plasma levels demonstrated when this agent is administered in combination with zoxazolamine is probably due mainly to an alteration in the metabolism of chlorzoxazone. In addition, evidence has been presented to support the theory that this modification in the metabolism of chlorzoxazone may be mediated through the formation of a molecular complex between the muscle relaxant compounds.

Prolongation of drug activity by mechanisms similar to that described in this study would be especially useful for those drugs which are absorbed in a limited portion of the gastrointestinal tract and therefore cannot be prolonged by a delayed release mechanism. Furthermore, many of the problems encountered in controlling the release of drugs and assuring their complete availability from the various sustained action forms would be greatly simplified in a system of this type.

SUMMARY AND CONCLUSIONS

Chlorzoxazone plasma levels were determined in humans, mice, and dogs after the oral administration of this compound alone, and in mice and dogs after administration of this agent in conjunction with zoxazolamine. In addition, the chlorzoxazone plasma levels in mice after intraperitoneal administration of the drug alone and in combination were determined.

The zoxazolamine plasma levels in mice and dogs were observed after oral administration of this compound alone and in combination with chlorzoxazone.

The brain levels of these compounds attained in mice after the oral administration of the drugs alone and in combination were also determined.

Formation of a molecular complex between the agents under study was demonstrated by means of solubility studies.

A prolongation of chlorzoxazone plasma levels was noted when this compound was administered simultaneously with zoxazolamine to mice or dogs. Evidence was presented to show that this prolongation probably results mainly from an alteration in the metabolism of chlorzoxazone. Furthermore, it is suggested that the proposed change in metabolism is mediated through the formation of a molecular complex *in vivo* between these muscle relaxant compounds.

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A Programmed Automated Film-Coating Process

By LEON LACHMAN and JACK COOPER

The design and operation of a programmed automated tablet coating process[1] is described. The electronics of the programmer, the baffle design for the coating pan, and the spray equipment used are illustrated and discussed. The advantages and superiority of this process as compared with the customary manual coating techniques are presented.

DURING THE PAST several years there has been a continuous and rapid movement away from the time-honored sugar coating to film coating of tablets. Considerable research time has been devoted to the development and testing of water- and gastric-soluble (1–11) and enteric (12–19) film-coating formulations. However, during this same period of time, little has been done to improve the process of applying the film coating onto tablets to insure reproducibility of the physical appearance and disintegrating characteristics of coating from batch to batch of tablets. The few attempts in this direction consisted of using a pneumatic gun to spray the tablets manually with coating solution (20) or the use of fluidized bed–coating techniques (21–23). Both methods, while being improvements over the customary manual technique of coating, still left something to be desired.

Most of the film coats presently on the market are of the water-soluble type which, in part, is the cause of the lack of progress in improving the coating process. Uniformity of water-soluble coating from batch to batch of tablets is of less importance than for enteric film coats. Satisfactory physical appearance and sufficient coverage to mask an unpleasant odor or taste are the major criteria for water- or gastric-soluble film coats. However, it is essential that the enteric film coating be uniform from batch to batch of tablets since the coated tablets must meet certain required disintegrating properties (U.S.P. XVI, p. 935) which are critically affected by film uniformity.

It is generally recognized that the human element is responsible for the variations that take place in batches of film-coated tablets. The thinness of the film coats, generally 1–10% of the uncoated tablet weight, cannot tolerate human variations or errors from batch to batch of tablets, which are known to occur in the customary sugar-coating technique. However, since sugar coats are usually equal to the weight of the uncoated tablet, the variations and errors due to the human factor become less meaningful.

In film coating, and more particularly in enteric film coating, it is imperative to remove the "art" from the coating process and place it on a more exact basis. This could most readily be accomplished by eliminating or minimizing the human factor in the coating process.

This report presents a detailed description of a programmed automated process for film coating of tablets recently developed in our laboratories which completely removes the human factor from the coating operation. In addition, data will be presented illustrating the superiority of this process over the customary manual method of tablet coating.

DESIGN AND EQUIPMENT

The schematic diagram in Fig. 1 illustrates the overall design and equipment involved in automating and programming the film-coating operation. Before detailing the various components of this process, a brief description of the operation and function of the programmer will be presented.

The coating process is controlled by a perforated

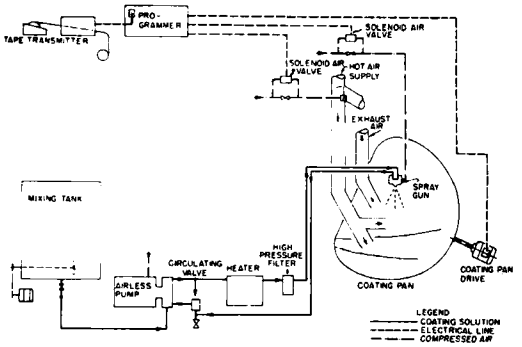

Fig. 1.—A schematic diagram of the design and equipment for the programmed automated coating operation.

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[1] Process patent pending.

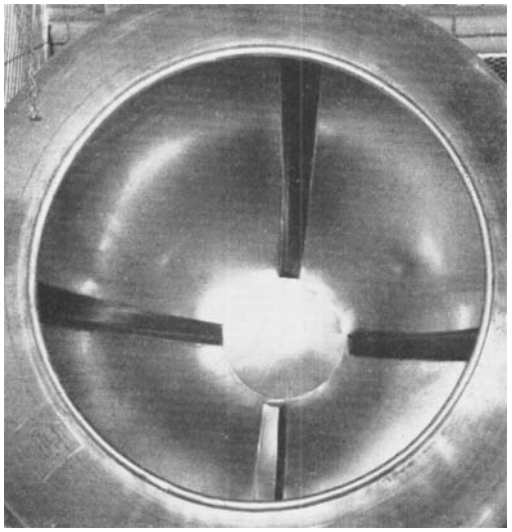

Fig. 2.—A photo depicting the baffle system.

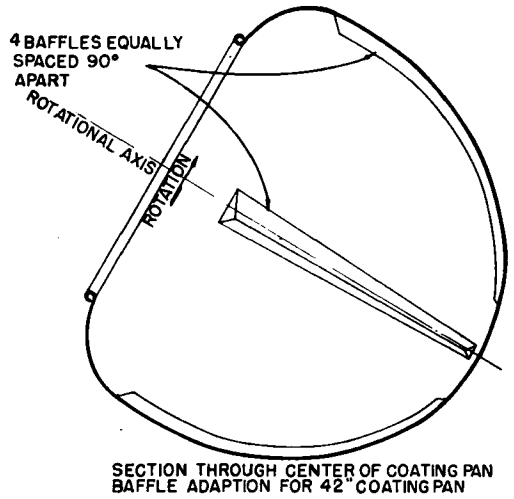

Fig. 3.—A schematic representation of the baffle design and placement in the coating pan.

tape traveling at a certain distance per unit time and transmitting its signals through a tape transmitter to the necessary relays in the programmer to activate the several components of the coating cycle, which include: (*a*) switching on coating-pan motor to start pan rotation; (*b*) regulating the pneumatic solenoid which opens and closes the automatic spray gun; (*c*) controlling a pneumatic solenoid which activates a single acting air cylinder that opens and closes the damper on the forced hot air duct; and (*d*) activating a latching relay terminating the coating operation.

In order to present a clear description of the design and equipment for this process, it has been divided into three segments which will now be discussed in detail.

Coating Pan, Forced Air, and Exhaust Ducts.—As mentioned earlier, the purpose of automating and programming the coating operation was to eliminate or reduce to a minimum any manual operations during the film-coating process. A phase of the coating operation where manual handling of the tablets is generally essential is subsequent to the addition of the coating solution. At this point the wet tablets tend to slide instead of tumbling. The coater usually places his hand into the pan and manually moves the tablets to aid the distribution of the coating solution until the tablets have partially dried and begin to tumble. In an effort to eliminate this manual handling phase of the coating operation, the influence of (*a*) pan rotational speed, (*b*) pan design, and (*c*) baffles placed into the pan on the tumbling action of tablets in the wet and dry state were investigated. Results from these tests indicated that the use of baffles of a particular design would provide the most suitable means for attaining this objective.

After considerable testing of different shapes, sizes, and numbers of baffles for use in a 42-in. pear-shaped stainless steel pan, the baffle system illustrated in Fig. 2 was found to be optimal. As is shown, the pan was divided into quadrants and a baffle was placed in each quadrant equidistant from one another. Figure 3 shows a schematic representation of the baffle designs and their placement in the coating pan. It is evident from this figure that the baffles have a gradual rise from the bottom to the top of the pan as well as slope in the direction of the pan's rotation.

The rise in baffle height from the inside to the outside of the coating pan was necessary to effect an even tumbling action throughout the pan, since the pan shape would cause the tablets to tumble at different speeds from the bottom to the top of the pan.

The slope of the baffles in the direction of the pan's rotation varies from 1 in. at the bottom to 2.5 in. at the top. This slope of the baffles is essential to prevent a too high carry over of tablets with a subsequent high fall causing irregular tumbling action and a possible focal point for tablet sticking and collection of coating solution and duster.

The baffles are 33 in. long, made of stainless steel, and fit the curvature of the pan. The baffles begin about 5 in. from the bottom center of the pan to about 10 in. from the top. Placing the baffles 5 in. from the center point of the pan gives a 10-in. diam. bottom area that is not baffled. This free area is necessary for correct tumbling action. The 10 in. at the top of the pan remain unbaffled for the same reason. If the baffles were extended at either end, the tablets no longer exhibit a smooth tumbling action, but rather a jumping action. It is also desirable to taper off both ends of the baffle gradually to enhance further smooth tumbling of the tablets.

Studies of the coating operation showed that sticking of tablets on the pan wall (when present) would radiate from the bottom center of the pan. Entering air is not sufficient to assist in drying this section of the pan wall because it is almost continuously covered with tablets. To eliminate this focal point for sticking, a Y attachment was developed for the forced hot air duct so that one part of the Y directed air to this focal point and the other part of the Y directed air onto the tablets. This is illustrated in Fig. 4 (the other duct shown in the pan is the exhaust duct which removes solvent vapors and dust).

Spray Equipment for Application of Coating Liquid and Duster.—With one of the human factors now eliminated, the other major variable was the

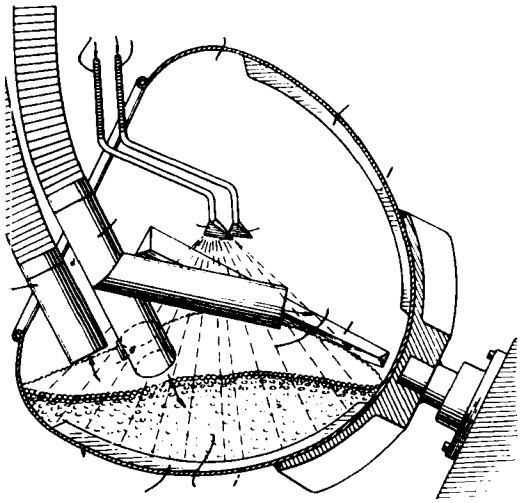

Fig. 4.—A schematic illustration of the Y attachment on the forced hot air duct.

application of the coating liquid and duster. In order to insure the addition of exact quantities of coating liquid and duster to the tablets at definite time intervals, this phase of the coating operation had to be placed on an automated basis and removed from the control of the tablet coater. To automate this operation, it was essential to develop an electronically controlled method of adding the coating liquid and duster. Spray guns for applying the coating liquid and duster seemed most feasible.

Existing methods for applying the coating liquid and duster to tablets consist of pouring a portion of coating liquid from a measuring container and a quantity of duster from a scoop. This method relies essentially on the tumbling action of the tablets for the distribution of coating liquid and duster. However, if the liquid and duster are added as a fine spray, the distribution of these coating materials on the tablets should be significantly improved compared to the hand method of addition. The reproducibility and uniformity of repeated coats can be accurately controlled through the use of a sprayed quantity of liquid and duster by regulating the duration of spray. This would in turn produce tablets exhibiting a more uniform and elegant film.

A spray process could be further improved by including the duster (*e.g.*, talc) in the coating liquid and applying the suspension as a spray, thereby eliminating the dusting step from the operation. This could produce four advantages: (*a*) talc in liquid suspension lends itself to easier fluidization than talc powder so that a reproducible quantity of material is sprayed per unit time; (*b*) using talc in liquid suspension permits a more uniform distribution of talc on the tablets being coated and consequently a thinner enteric film; (*c*) a reduction or elimination of the inherent dust problem of a fluidized talc spray resulting when talc in the form of a liquid suspension is used; and (*d*) the elimination of one operation from the coating cycle.

For film coating it seemed that a spray gun which gives an *airless spray* would be most desirable. The term *airless spray* is used to refer to a spray coating method in which a coating material is atomized by ejecting it from a spray nozzle by a hydraulic pressure imposed on the coating material itself.

The conventional *air spray method* atomizes the coating material by striking it with a high speed jet of compressed air at the end of the atomizing gun chamber. Because air is so light, it takes a tremendous amount of air moving at a high speed to atomize properly a viscous material. The stream emerging from an air atomizing gun is actually a high speed air current in which a small fraction of liquid is dispersed, generally about 1% or less. The liquid droplets are blown forward at high speeds and the atomizing air expands and rebounds causing much of the coating liquid to be blown away from the material to be coated. In addition, as the atomizing air expands, it also cools. This effect, coupled with the cooling effect of solvent evaporation, can lower the temperature of the atomized droplets far below the temperature of the surrounding air. If the temperature were to drop below the dew point, atmospheric water could condense on the particles and could cause the particles to deposit irregularly on the tablets, exhibit poor flow-out properties, cause the film to take on a blush appearance, and possibly rub off from the tablets.

However, the airless spray gun gives a completely different type of spray. Here, since no atomizing air is used, the coating material itself is discharged from the spray nozzle with possibly a minimum amount of air. The droplets move forward by their own momentum. The atmosphere between the spray gun and material to be coated resists the movement of droplets which are appreciably slowed down by this air resistance. Further, the energy released at the nozzle of an airless gun is only a fraction of that required in the air atomizing gun with a consequent drastic reduction in rebound or overspray from the material being coated. In addition, by the use of the airless spray gun, the amount of solvent vapors escaping from the coating pan is kept at a minimum and substantially below explosive and health limits. The airless spray gun would be particularly necessary if a duster were to be sprayed onto the tablets. One could just imagine the dust problem that would result if an air atomizing gun was used.

Figures 5 and 6 depict the relative spray characteristics for an air and airless spray gun using a coating liquid of the same viscosity. It is readily evident that a significantly greater overspray and rebound takes place for the air spray system as well as requiring a higher energy for atomization. Figure 6 shows that when the liquid is heated before spraying, a reduction of atomization energy takes place with the airless system requiring about one-third and the air system about two-thirds that of the cold spray.

Since it would be advantageous for the spraying system to be used for automating the coating process to have maximum versatility, a thorough investigation was made of the various spray equipment available. A unit of particular suitability and versatility was found in a Nordson airless spray-coating unit. This spray system atomizes the coating liquid, heated or unheated, by ejecting it from the spray nozzle through a hydraulic pressure imposed on the coating material itself.

The design and components of the spray system are schematically presented in Fig. 7. In this system the coating liquid in the supply tank is drawn up the siphon tube to the pump and pressurized, pushed

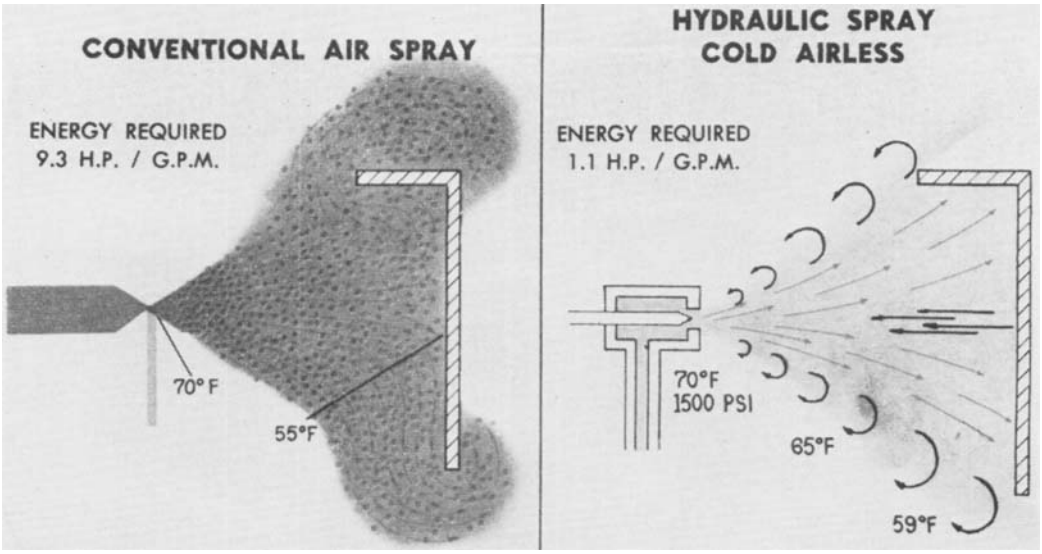

Fig. 5.—The relative spray characteristics of a liquid at room temperature for an air and airless spray system.

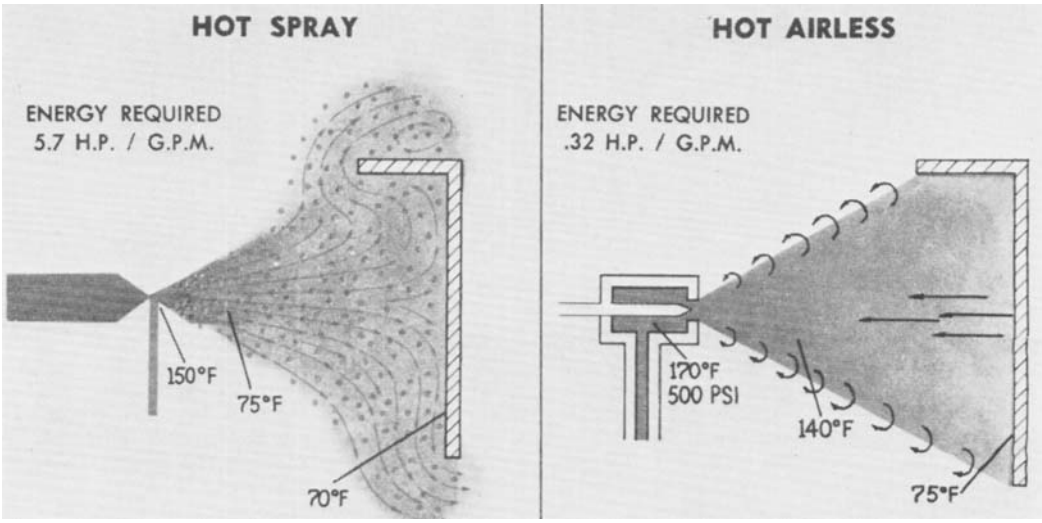

Fig. 6.—The influence of heated liquid on the spray characteristics of an air and airless system.

through the heater (which is specially designed to withstand the high hydraulic pressures needed for atomization), the high pressure filter (which removes particles in liquid suspension that are greater than the nozzle orifice), and then flows to the spray guns available for atomization. The Nordson automatic guns are of a special design making it possible to open and close the gun in less than *one-hundredth* of a second.

This spray system is designed to keep the liquid in the unit circulating to and from the gun when the gun is and is not spraying. When the system is not spraying, the coating liquid travels from the gun through a return line and then through a recirculating valve permitting a controlled amount of liquid to pass into the suction part of the pump. From here the liquid is repressurized for circulating through the system. This assures a continual flow of material through the system; the coating material

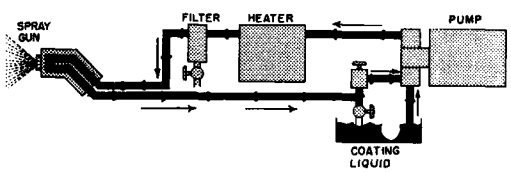

Fig. 7.—Design and components of the airless spray system.

at the nozzle is always at the correct temperature and ready to be sprayed whenever the gun is triggered. In addition, it prevents material in suspension from settling out as well as eliminating possible clogging of the orifice tip due to particle accumulation resulting from the loss of volatile solvents. When the gun is triggered and coating liquid forced out of the nozzle, a similar amount of

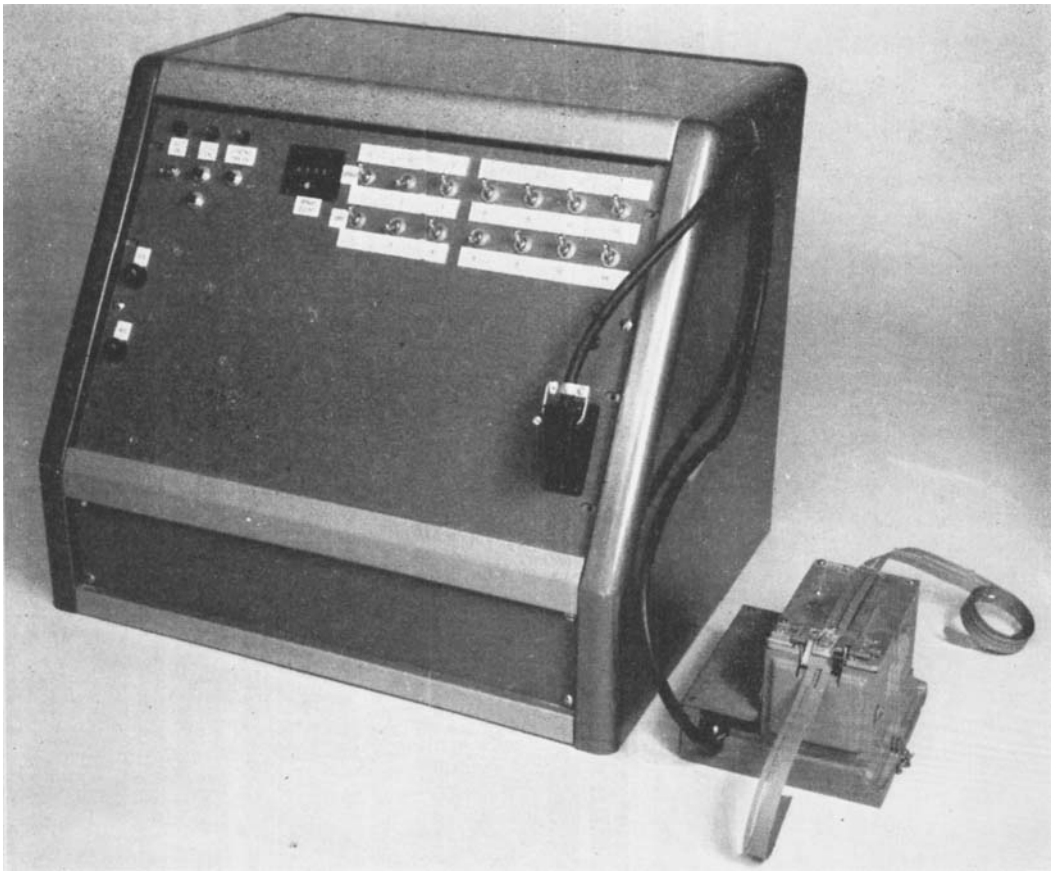

Fig. 8.—Photo of programmer used to automate coating operation.

liquid is withdrawn from the supply container and added to the recirculating pump. Therefore, recirculation is maintained even when the system is spraying. In this system coating liquid does not flow back into the supply container. If it did, then the entire supply of coating material would eventually become heated causing evaporation of the low boiling solvents in the coating material.

In our studies, carbide tip nozzles giving a flat spray pattern were used. The flow rate of the liquid through the nozzle is dependent upon the viscosity and temperature of the liquid, hydraulic pressure, and orifice measurements. However, the major controlling factor for flow rate and spray angle is the nozzle geometry and dimensions.

Automation and Programming of Film-Coating Operation.—With the spray equipment chosen and the coating pan modified to permit tablets to tumble when wet or dry, the final step was to design a programmer to automate the coating operation and remove the human element completely from the coating process. After studying various ways to program the film-coating operation, a punched tape traveling at a certain distance per unit time and transmitting its signals through a tape transmitter to the necessary relays to activate the several elements of the coating cycle appeared to be the simplest to design, install, and operate. In addition, such a system could be built with binary circuitry giving maximum flexibility relative to making changes in the time allotted to each sequence of the cycle. When the changes in cycle time involve only the repetitive spray-dry cycles of the coating operation, the same tape can be employed and the changes made on the programmer by adjusting the toggle switches controlling the binary circuitry. Only when the irregular parts of the coating cycle require changing is it necessary to use a new tape. Therefore, such a programming system can readily be used for various tablet formulations requiring different coating cycles. Any programming arrangement can be obtained with this system by modifying the tape or modifying the binary circuitry on the programmer. Paper tapes can generally be used between 50 to 100 times before replacement. If greater permanency is desired, mylar tapes can be used.

Figure 8 shows the programmer which consists of a Western Union tape transmitter designed for 5-unit code transmission using perforated tape with in line feed holes. The perforated tape travels at a certain distance per unit time transmitting its signals through the tape transmitter to the necessary relays in the control box which activates the several elements of the coating cycle. The timer in the control box regulates the residence time in the transmitter for each hole in the tape.

As the tape is pulled over the code pins of the tape transmitter by the feed wheel traveling at a certain rate, signals are transmitted to the relays in the

control box activating the various functions of the coating cycle and (*a*) starts coating pan rotation by switching on motor through a relay and step down transformer; (*b*) regulates the three-way pneumatic solenoid which opens and closes the automatic spray gun used to spray doses of coating suspension; (*c*) controls a pneumatic solenoid which activates a single acting air cylinder which opens and closes the damper to the forced hot air duct, supplying the hot air for drying the tablets; and (*d*) activates a latching relay which terminates the coating cycle and shuts off the programmer.

By employing this programmed automated process of tablet coating, the volume of coating suspension and, if needed, the weight of talc can be controlled by the length of time of spray at constant hydraulic pressure and nozzle size. Consequently, it should be possible to obtain readily reproducible coatings from batch to batch of tablets.

EXPERIMENTAL

The influence of the human factor on the disintegration properties, uniformity, and physical appearance of enteric films will be illustrated with data obtained from coating $^{15}/_{32}$-in. modified ball tablets weighing 1.06 Gm. by both the customary coating techniques and by the programmed automated process. In both instances the coating operation was performed on 85 Kg. batches of tablets in the stainless steel 42-in. pear-shaped baffled coating pans. For the automated process the tablets were coated with a suspension of 10% talc in a 10% solution of cellulose acetate phthalate containing diethyl phthalate as plasticizer and a solvent system of anhydrous ethanol-acetone, while for the customary technique the same coating solution was used except that the talc was added as a duster between coats instead of in suspension.

In the automated process the suspension was sprayed at a temperature of 50° and at a flow rate of about 1000 ml./minute. The hot air flowing into the pan to dry each coat was at 47°. The coating cycle employed was (*a*) an initial 90-second spray and 120-second dry period; (*b*) then repetitive cycles of 15 seconds consisting of 3-second spray and 12-second drying periods; (*c*) at the end of the repetitive cycles, a 5-minute drying period is permitted in the pan as the tablets tumble; (*d*) then the tablets are placed in an oven at 40° for thermosetting of the film coating and removal of residual solvents.

The normal coating procedure is the term applied to the procedure that was used in our Production Division and consisted of (*a*) manually spraying a quantity of coating solution onto the tablets, (*b*) permitting the tablets to tumble for a prescribed period of time, (*c*) manually dusting on a quantity of talc, (*d*) allowing the tablets to tumble to permit distribution of the talc, and (*e*) directing forced heated air onto the tablets for drying the coatings. These steps are repeated until the desired coating is obtained. At the completion of the coating operation the tablets are removed from the coating pan and placed into a circulating-air oven maintained at 40° to thermoset the film.

By removing samples of tablets during the programmed automated coating operation it was found that the tablets develop enteric properties between 150–200 repetitive cycles. Additional coatings within definite limits provide a safety margin for disintegration properties and improved appearance. Table I shows comparative data of film weight and film uniformity from batch to batch of tablets prepared by the automated process after 200 and 350 repetitive cycles (called end coating) and tablets prepared by customary coating techniques. It is readily evident from this table that it is possible to obtain enteric film coated tablets by the automated spray process with film coatings of about one-third the weight of those prepared by customary coating techniques. In addition, the range of mean coating weights for the 200 and 350 repetitive cycle (called end coating) coated tablets are approximately one-fourteenth and one-sixth that of tablets coated by customary manual coating techniques.

TABLE I.—MEAN WEIGHTS OF COATING OBTAINED BY AUTOMATED AND NORMAL COATING TECHNIQUES

Batches	Mean Coating Weights— 200 Coats	End Coating
Normal A	...	0.1007
Normal B	...	0.0914
Normal C	...	0.0736
Normal D	...	0.1269
Overall Mean	...	(0.0981)
Range of Means	...	0.0533
Automated A	0.0358	0.0594
Automated B	0.0341	0.0639
Automated C	0.0365	0.0580
Automated D	0.0378	0.0671
Automated E	0.0370	0.0639
Overall Mean	(0.0362)	(0.0623)
Range of Means	0.0037	0.0091

Table II summarizes the standard deviations for the uncoated and coated tablets used for the normal and automated processes. Comparing the overall mean standard deviation of uncoated tablets used for the normal and automated process of coating, it was found that they do not differ significantly from one another. However, in comparing the overall mean standard deviation for the tablets coated by the normal techniques with those coated by the automated process it is found that the overall standard deviation for the tablets coated by the automated process is significantly less than that for the tablets coated by the normal process.

TABLE II.—STANDARD DEVIATIONS OF MEAN WEIGHTS OF UNCOATED AND COATED TABLETS

Batches	Uncoated Std. Dev.	End Coating Std. Dev.
Normal A	0.0050	0.0203
Normal B	0.0064	0.0222
Normal C	0.0091	0.0288
Normal D	0.1110	0.0315
Overall Mean	(0.0079)	(0.0257)
Automated A	0.0106	0.0098
Automated B	0.0106	0.0130
Automated C	0.0106	0.0100
Automated D	0.0102	0.0095
Automated E	0.0059	0.0100
Overall Mean	(0.0089)	(0.0105)

The time required for the automated process to give tablets coated by 200 repetitive cycles is about 60 minutes and by 350 repetitive cycles, about 90 minutes.

From our experiences with this programmed automated process of coating it became readily apparent that it offered a number of advantages over the manual method of coating, the most significant ones being:

Tablets from a particular pan batch show uniform coatings relative to physical appearance and disintegrating properties.

Reproducibility of coating from batch to batch of tablets is insured.

The more even film coating deposited by this process produces enteric coated tablets with one-third or less the film weight necessary for manual coating procedures.

Using this process with the repetitive short spray-dry cycles of coating, no coating build-up on the pan wall takes place. This permits the continuous coating of batches of tablets without the need of washing the pans between batches.

The possibility of pimples forming on the coating is eliminated because no coating build-up on the pan wall results.

The time required to obtain enteric film coated tablets by the automated process is at least half that required for the manual process.

A manifold increase in production capacity is possible through this process of film coating.

One operator will be able to supervise the coating of many batches of tablets per day.

It is unnecessary to use an experienced tablet coater with this system of film coating.

This method of coating is adaptable for use with water-soluble films as well as sugar coating.

SUMMARY

A programmed automated process for film coating has been developed which is capable of concurrently coating multiple pan batches of tablets containing 85–100 Kg. of tablets per pan.

As a result of eliminating the human element from the coating operation and consequently the so-called "art" of tablet coating, film-coated tablets prepared by the automated process show close reproducibility of coating from batch to batch of tablets as well as greater uniformity of coating within batches of tablets.

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Notes

Concentric Double Electrolytic Junction Calomel Reference Electrode

By NORMAN ADLER

Aqueous calomel reference electrodes are frequently unsuitable for use in titrations in acetic acid or acetic anhydride as they often lead to erratic potentials and drifting, irreproducible end points (1–4). These difficulties have been attributed to contamination of the electrode (3), to insufficient electrode-solution interfacial contact (1), and to changes in the liquid junction potential as the electrolyte flows from the electrode to the solution (4). Asbestos fiber-type electrodes, notoriously troublesome in nonaqueous titrations (4, 5), may be particularly prone to contamination. As the titration solution (often containing perchlorate ion) diffuses into the fiber, it mixes with saturated aqueous potassium chloride. Less soluble potassium perchlorate may then be precipitated, thereby disturbing or breaking the electrolytic contact. Depending on the titration medium, the electrolyte may also be precipitated. A crust of precipitate has been observed to form at the tip of the fiber in contact with the titration medium (6). Fiber electrodes that become shorted or erratic during nonaqueous titrations may often be rejuvenated by soaking overnight in water.

The difficulties with aqueous electrodes may also be attributed to the nature of the electrolyte and the solvent used. The addition of water to the titration medium changes the nature of the system, often deleteriously (3). Water and the occasionally used methanol (7, 8) are not suitable for acetic anhydride systems as they undergo acid-catalyzed

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From our experiences with this programmed automated process of coating it became readily apparent that it offered a number of advantages over the manual method of coating, the most significant ones being:

Tablets from a particular pan batch show uniform coatings relative to physical appearance and disintegrating properties.

Reproducibility of coating from batch to batch of tablets is insured.

The more even film coating deposited by this process produces enteric coated tablets with one-third or less the film weight necessary for manual coating procedures.

Using this process with the repetitive short spray-dry cycles of coating, no coating build-up on the pan wall takes place. This permits the continuous coating of batches of tablets without the need of washing the pans between batches.

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acetylation reactions. Chloride salt electrolytes are also potentially troublesome.

When the titration medium contains mercuric acetate, the alkali chloride electrolyte flowing into the solution is quantitatively converted to a strong base (3). In the absence of mercuric acetate, titrable base is formed less rapidly by the volatilization of hydrochloric acid (9, 10). In both cases the effect is manifested by a slow drift of the E.M.F. back to the alkaline side of the end point and leads to erroneously high results. Since the rate of base production is not appreciable, the effect may be noticed only in the end point region of the titration where the rate of change of potential with acid concentration is at a maximum.

In view of these effects many workers (6, 11) have recommended either modified calomel electrodes or alternate electrode systems for use as the reference electrode in nonaqueous titrimetry. The modifications of the calomel electrode usually consist either of using various alternate electrolyte-solvent combinations (2, 7, 8, 12) in the built-in salt bridge of commercial calomel electrodes (single junction type), or of immersing the aqueous calomel electrode in an external nonaqueous electrolytic bridge (double junction type). Various geometries for the external bridge chamber have been proposed (1, 4, 5, 11–13). The double junction type, although more complex, permits simpler trouble shooting in the event of electrode difficulties. Their higher resistance is ordinarily not a drawback if used with the high impedance pH meters currently available. In fact, Warner and Haskell (4) have successfully used acetic acid without added electrolyte as the solvent in the second bridge.

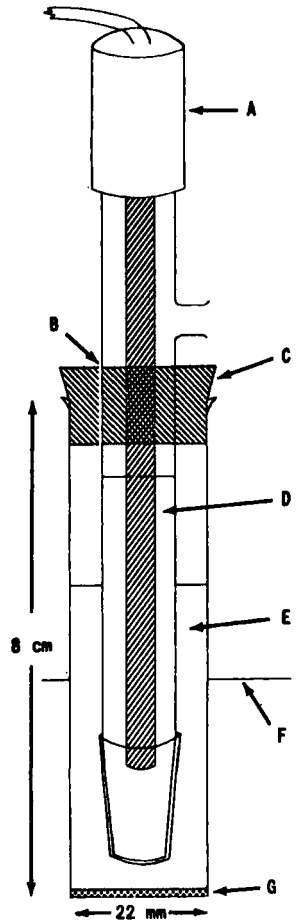

Fig. 1.—Concentric double electrolytic junction calomel reference electrode: *A*, Beckman sleeve-type calomel electrode, No. 1170–71; *B*, vent; *C*, stopper; *D*, saturated aqueous potassium chloride; *E*, saturated solution of potassium perchlorate in acetic acid; *F*, maximum insertion of electrode into solution; and *G*, porous disk.

A convenient version of the double junction type is shown in Fig. 1. It consists essentially of a simplification of the Warner and Haskell design (4) to avoid the necessity of a special titration cell. The compactness of the concentric tube design permits this electrode to be used as easily as an ordinary calomel electrode in the conventional two electrode holders supplied with commercial pH meters. For titrations in poorly conducting solutions where the distance between electrodes may be important (14), the outer bridge may be further modified by placing the porous frit on the side of the tube facing the glass electrode. When not needed for nonaqueous work, the aqueous calomel electrode may be easily removed, and, after rinsing, immediately used for aqueous titrations.

The porosity of the fritted disk (Corning Glass No. 37730, Allihn tube with fritted disk, 30F, 20 mm. diam., 100 mm. height, is cut flush with the disk and to a height of 8 cm.) is adjusted by flame treatment to give a flow rate of less than one drop/hour for a 2 inch head of electrolyte. Acetic acid saturated with potassium perchlorate is used as the electrolyte in the outer bridge. Introduction of reasonable amounts of this electrolyte into the titration system is without effect. As indicated in Fig. 1, the relative levels of the electrolytes in relation to the solution are arranged to give a positive outward flow, thus minimizing the possibility of electrode contamination by inward diffusion of the titration medium. Monthly changes of inner and outer electrolytes are usually sufficient to keep the electrode working properly. In use, the electrode equilibrates rapidly to give stable potentials of the same magnitude as obtained for other calomel reference electrodes. (A fresh aqueous fiber type, a sleeve type filled with lithium chloride in acetic acid, and the above type, gave the same potential within 10 mv. when measured against the same glass electrode in the same solution.) The concentric tube electrode has been in satisfactory use for many years in this laboratory.

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Low Molecular Weight Carbohydrates in *Sargassum natans* from Puerto Rico

By NOEMÍ G. MARTÍNEZ NADAL, LUZ V. RODRÍGUEZ, and CARMEN CASILLAS

***d*-Mannitol, *d*-mannitol monoacetate, and an unidentified carbohydrate were isolated from *Sargassum natans*.**

PRELIMINARY tests carried out in this laboratory indicated that an ether extract of *Sargassum natans* exhibited antibiotic properties (1). To obtain further data, aqueous and alcoholic extracts of this alga were prepared and tested for antibiosis. Negative results were obtained.

Since no work is reported on the polysaccharides found in *Sargassum natans*, we separated and identified some of the carbohydrates present in the alcoholic extract of this alga. The presence of *d*-mannitol, *d*-mannitol monoacetate, and unknown carbohydrate were evidenced.

EXPERIMENTAL

A chemical analysis of dried *Sargassum natans* from Puerto Rico gave the following results: 1.23 Gm. ether extract, 74.21 Gm. carbohydrates, 14.21 Gm. fiber, 0.77 Gm. nitrogen, 4.81 Gm. protein, 33 mg. tryptophan, 44 mg. methionine, 174 mg. lysine, 19.75 Gm. ash, 289 mg. calcium, 57.2 mg. phosphorous, 57.4 mg. iron, 0.01 mg. carotene, 0.31 mg. riboflavin, 1.46 mg. niacin, 3.70 mg. ascorbic acid, 326 calories.

An extract prepared by continuous extraction of 100 Gm. of dried alga, with 95% ethanol for 24 hours, on cooling, deposited about 3% of a white crystalline precipitate (ppt. No. 1).

The alcohol was removed from the rest of the sample under reduced pressure. The residue consisted of a crystalline, brownish mass, with an oily layer, which was removed by washing with ether. Several recrystallizations of this mass from alcohol gave ppt. No. 2. A small amount of ppt. No. 3 was obtained by the evaporation of cold alcoholic extracts.

Osazones, hydrazones, and semicarbazones were prepared of precipitate No. 1 and 2.

A second batch of 800 Gm. of dried alga was extracted for 67 hours with ether, followed by 71 hours of alcoholic extraction. A 19.8 Gm. quantity of ppt. No. 1 was obtained by decantation of the alcoholic extract. After concentration to dryness, the residue was dissolved in water and filtered. Lead acetate was added to the filtrate, the precipitate formed was removed, and the excess of lead was precipitated with hydrogen sulfide. After evaporation of the solution to dryness, the residue was dissolved in 1% ethanol and absorbed on a carbon Celite column (35 × 4.5 cm.) which was eluted with aqueous ethanol (1–15%). From 30–40 μl. of each fraction was investigated by paper chromatography prior and after hydrolysis with 0.1 *N* hydrochloric acid. Ethyl acetate:acetic acid:water (3:1:3 v/v) was used as solvent. The indicator spray used on monosaccharides was ammoniacal silver, Hough's method (2) and modified method of Trevelyn, *et al.* (3). The sugar alcohols were spotted by bromcresol purple reagent and appeared as yellow spots on a blue background owing to the pH change of the sugar-borate complex of Bradfield (4) and Hackmann (5).

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The work was carried out at the Research Center, College of Agriculture and Engineering, University of Puerto Rico, Mayaguez.

RESULTS

Sargassum natans is a brown alga, whose chemical composition revealed it to be rich in carbohydrates and low in nitrogen. It grows abundantly on the coasts of Puerto Rico.

Three pure compounds were obtained from the alcoholic extraction of this alga. Ppt. No. 1, m.p. of over 300°, contained carbon, hydrogen, and oxygen, reduced the silver nitrate sodium ethoxide reagent, gave a positive carbazol reaction for carbohydrates, did not reduce Fehling's solution and reacted with semicarbazide hydrochloride to form a compound, m.p. 119–120°. Its chromatographic strip resembles in R_f and appearance a cyclitol.

Ppt. No. 2, identified, as *d*-mannitol, by paper chromatography, and by derivatives of phenylhydrazine and semicarbazide hydrochloride, was confirmed by microanalysis.[1]

Ppt. No. 2 (pure) Found: C = 39.47; H = 7.79. Calculated as *d*-mannitol: C = 39.53; H = 7.74. Phenylhydrazine derivative of ppt. No. 2. Found: C = 64.51; H = 6.17; N = 18.80. Phenylhydrazine derivative of pure *d*-mannitol. Found: C = 64.55; H = 6.19; N = 19.00. Semicarbazide derivative of ppt. No. 2. Found: C = 20.49; H = 5.6; N = 46.55. Semicarbazide derivative of pure *d*-mannitol. Found: C = 20.46; H = 5.08; N = 46.47.

Ppt. No. 3, m.p. 124–126°, which was identified by paper chromatography as *d*-mannitol monoacetate was confirmed by microanalysis as $C_8H_{16}O_7$. Found: C = 42.8; H = 7.19. Calcd.: C = 42.9; H = 7.05. The hexacetate formed from the hydrolyzed sample of this monoacetate was also confirmed by microanalysis. Found: C = 50.84; H = 5.92. Calcd.: C = 50.21; H = 5.76.

SUMMARY

1. Chemical analysis of *Sargassum natans* indicated it to be rich in carbohydrates and low in nitrogen.

2. Three natural products were separated by solubility ratios and column chromatography from *Sargassum natans* from Puerto Rico.

3. *d*-Mannitol and *d*-mannitol monoacetate were identified among the three products obtained.

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[1] All microanalyses were performed by Dr. L. I. Duiguid at St. Louis, Mo.

Investigation of Pantothenyl Alcohol in Experimentally Induced Intestinal Atony

By THOMAS A. LYNCH, WILBUR L. HIGHLEY, and ALBERT G. WORTON

Pantothenyl alcohol (10 mg./Kg.) given subcutaneously significantly increased gastrointestinal motility at 15 and 18 hours in mice and at 20 and 24 hours in rats following cecectomy, but exerted no effect in normal animals. Optimal conditions for evaluating pantothenyl alcohol consisted of measuring the 30- and 45-minute progression of an orally administered charcoal meal through the gastrointestinal tract, 14 to 24 hours following cecectomy.

BLY, *et al.* (1), in 1943 and Jurgens and Pfaltz (2) in 1944 were the first to report the occurrence of intestinal atony in pantothenic acid deficient animals. These observations lead to clinical investigations by Kareha, *et al.* (3), Haycock, *et al.* (4), Stone, *et al.*, (5), Frazer, *et al.* (6), and Nardi and Zuidema (7), who reported pantothenyl alcohol to be effective in preventing and treating intestinal atony and gaseous abdominal distention in postsurgical patients. In 1950 Lipmann, *et al.* (8), established pantothenic acid as an integral part of coenzyme A. The *in vivo* formation of acetyl coenzyme A and the acetate transfer to choline, elaborate the transmitter substance, acetylcholine, required for intestinal motility. Unna and Greslin (9) reported pantothenic acid to be pharmacologically inert and relatively nontoxic. Polacek, *et al.* (10), using a double blind technique, were able to significantly reduce the duration of experimental post-surgical ileus in mongrel dogs. In this study a method of evaluating pantothenyl alcohol or similarly acting agents in small laboratory animals is presented.

EXPERIMENTAL

Mice (15–36 Gm.) and rats (60–250 Gm.) were anesthetized with ether, the abdominal cavity opened, and the cecum tied off and removed. Following the closure of the abdomen by suturing, the drug was administered subcutaneously. At various intervals following cecectomy, a 12.5% powdered charcoal suspension in 1% methyl cellulose solution was given orally using a dosage equivalent to 2.5% of the body weight. At a specific time following charcoal administration, the animals were decapitated, the entire gastrointestinal tract was removed, and the distance that the charcoal meal had progressed was expressed as per cent of the total length of the gastrointestinal tract.

RESULTS AND DISCUSSION

Pantothenyl alcohol (10 mg./Kg.) given subcutaneously just after cecectomy did not alter the 12 to 18 hours passage of charcoal meal, which progressed only 60 to 100% the length of the gastrointestinal tract as determined in 106 black (C-57)[1] male mice (15–36 Gm).

Using a 30-minute charcoal passage period, pantothenyl alcohol (1000 mg./Kg.) did not affect gastrointestinal motility in normal Swiss female mice (15–30 Gm.) at 1 to 14 hours, but increased motility at 14 hours in cecectomized mice ($P < 0.10$).

Gastrointestinal charcoal passage rates at 45 minutes in mice, as shown in Table I, indicate that the optimal effect of pantothenyl alcohol occurs between 11 and 18 hours following cecectomy. Pantothenyl alcohol significantly enhanced gastrointestinal activity at 15 hours ($P < 0.05$) and at 18 hours ($P < 0.001$).

TABLE I.—EFFECT OF PANTOTHENYL ALCOHOL ON FORTY-FIVE MINUTES GASTROINTESTINAL PASSAGE OF CHARCOAL IN CECECTOMIZED MICE[a]

Time, Hr.	Total No. Mice	Control[b], % and S.E.[e]	Treated[c], % and S.E.[e]	P[d]
11	20	39.6 ± 6.0	45.5 ± 7.0	N.S.
15	16	45.1 ± 8.0	72.5 ± 5.0	<0.05
18	35	36.1 ± 2.9	58.9 ± 3.9	<0.001

[a] Black male mice (C-57) weighing 15–36 Gm. [b] Control given 8 ml./Kg. of normal saline subcutaneously immediately after cecectomy. [c] Pantothenyl alcohol 8 ml. (10 mg.)/Kg. given subcutaneously immediately after cecectomy. [d] Probability level on significance based on *t* test. [e] Mean per cent and standard error of the total distance that charcoal passed through the G. I. tract in 45 minutes.

When 45-minute charcoal passage rate was used in rats, pantothenyl alcohol enhanced gastrointestinal motility optimally at 20 hours, an increase of 72% over controls as shown in Table II. A 35% increase by pantothenyl alcohol was observed at 24 hours. No significant change was observed at 18 hours.

TABLE II.—EFFECT OF PANTOTHENYL ALCOHOL ON FORTY-FIVE MINUTES GASTROINTESTINAL PASSAGE OF CHARCOAL IN CECECTOMIZED RATS[a]

Time, Hr.	Total No. Rats	Control[b], % and S.E.[e]	P.A.,[c] % and S.E.[e]	P[d]
18	9	30.6 ± 4.3	24.4 ± 4.5	N.S.
20	10	27.2 ± 3.8	46.8 ± 1.7	<0.01
24	17	39.9 ± 3.7	53.8 ± 5.4	<0.05

[a] Male Wistar rats (75–250 Gm.). [b] Normal saline 8 ml./Kg. given subcutaneously immediately after cecectomy. [c] Pantothenyl alcohol 8 ml. (10 mg.)/Kg. given subcutaneously immediately after cecectomy. [d] Probability level of significance based on *t* test. [e] Mean per cent and standard error of the total distance that charcoal passed through the G.I. tract in 45 minutes.

Pantothenyl alcohol did not reduce the time required for a charcoal meal to pass through the entire gastrointestinal tract as determined in 27 cecectomized male Wistar rats (60–68 Gm.).

As illustrated in Figs. 1 and 2, the pattern of

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[1] Rockland Farms, New York, N. Y.

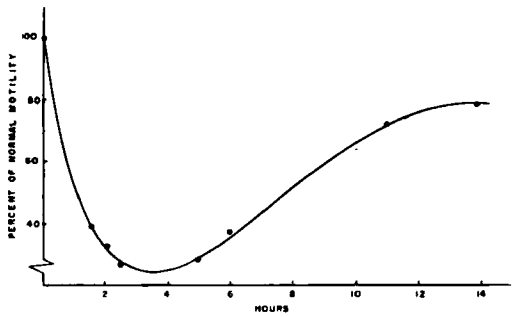

Fig. 1.—Pattern of surgically induced gastrointestinal atony and restoration in cecectomized mice as determined by the 30-minute passage of orally administered charcoal. Normal motility = 100%.

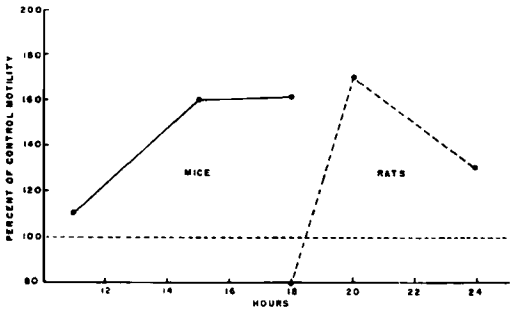

Fig. 2.—Pantothenyl alcohol (10 mg./Kg.) effect in the response phase of surgically induced atony as determined by the 45-minute passage of charcoal. Control motility in cecectomized animals = 100%.

upper gastrointestinal motility in regard to surgical cecectomy and pantothenyl alcohol effect appears to be: (*a*) an atony phase, (*b*) a restoration phase, and (*c*) a pantothenyl alcohol-effect phase.

The results suggest that upper gastrointestinal motility as measured by 30 or 45 minutes charcoal passage rates at 14 to 24 hours postoperatively in mice and rats is optimal for evaluating pantothenyl alcohol or similarly acting compounds.

The data presented tend to confirm in part the work of Unna and Greslin (9) in that pantothenic acid does not exert a direct pharmacologic effect in normal animals, and substantiates the report of Polacek, *et al.* (10), in which pantothenyl alcohol hastened the return of normal gastrointestinal motility. The difference in response of normal and post-surgical animals suggests that surgically induced atony may be related to a depression in function of the pantothenate-coenzyme A-acetylcholine biochemical pathway. Pantothenyl alcohol may act by accelerating this function in the post-surgical animal.

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Quantitative Evaluation of Surface Anesthetics in Albino Mice

By W. R. JONES and L. C. WEAVER

The corneal reflex of the albino mouse is satisfactory for the quantitative determination of relative potency of local anesthetics. It was found that dyclonine was more active, benzonatate equally active, and procaine less active than cocaine.

THE CORNEAL reflex of cats (1), dogs (2), rabbits, and guinea pigs (3) has been used for the detection of local anesthetic activity. Pittenger (1) suggested the use of the cornea of cats for the qualitative detection of local anesthetic action. However, Munch (2) concluded that cats are not as suitable as rabbits because of greater individual differences in activity and because of greater reluctance on the part of the test animal to permit the touching of the cornea at frequent intervals. He also concluded that qualitative tests made upon the cornea of dogs offer no particular advantage. The corneal reflex in the rabbit has not been successfully standardized and has not withstood statistical examination (3). Although Chance and Lobstein (3) report that guinea pigs show a more regular reflex response, both normally and when anesthetized, than do rabbits, Bulbring and Wajda (4) found that guinea pigs frequently fail to blink even if the cornea is not anesthetized. Regardless of the relative advantages of guinea pigs *vs.* rabbits, however, Chance and Lobstein (3) introduced a method which made possible a quantitative comparison of local anesthetics.

When preliminary tests indicated that the albino mouse might be a satisfactory animal for the bioassay of local anesthetics, an experiment was carried out to determine a dose-response curve for cocaine, procaine, dyclonine, and benzonatate. The ac-

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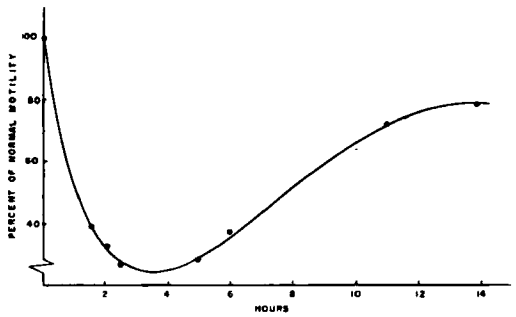

Fig. 1.—Pattern of surgically induced gastrointestinal atony and restoration in cecectomized mice as determined by the 30-minute passage of orally administered charcoal. Normal motility = 100%.

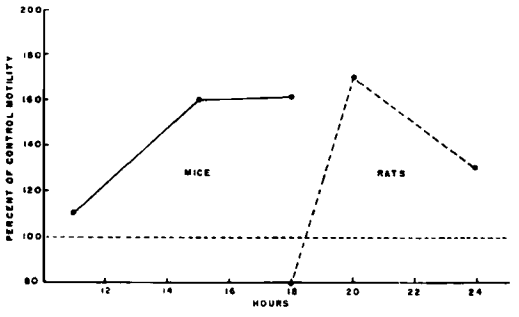

Fig. 2.—Pantothenyl alcohol (10 mg./Kg.) effect in the response phase of surgically induced atony as determined by the 45-minute passage of charcoal. Control motility in cecectomized animals = 100%.

upper gastrointestinal motility in regard to surgical cecectomy and pantothenyl alcohol effect appears to be: (*a*) an atony phase, (*b*) a restoration phase, and (*c*) a pantothenyl alcohol-effect phase.

The results suggest that upper gastrointestinal motility as measured by 30 or 45 minutes charcoal passage rates at 14 to 24 hours postoperatively in mice and rats is optimal for evaluating pantothenyl alcohol or similarly acting compounds.

The data presented tend to confirm in part the work of Unna and Greslin (9) in that pantothenic acid does not exert a direct pharmacologic effect in normal animals, and substantiates the report of Polacek, *et al.* (10), in which pantothenyl alcohol hastened the return of normal gastrointestinal motility. The difference in response of normal and post-surgical animals suggests that surgically induced atony may be related to a depression in function of the pantothenate-coenzyme A-acetylcholine biochemical pathway. Pantothenyl alcohol may act by accelerating this function in the post-surgical animal.

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TABLE I.—POTENCY OF SEVERAL SURFACE ANESTHETICS IN ALBINO MICE

	Dyclonine	Benzonatate	Procaine	Cocaine
ED_{50} (mg./ml.)	0.27	2.45	34.5	2.45
Fiducial limits P — 0.95	0.16–0.47	1.64–3.68	23.0–51.8	1.23–4.90
Slope	2.72	2.03	2.11	2.08
Fiducial limits P — 0.95	1.13–6.53	1.08–3.82	0.75–5.91	1.16–3.74

tivity of these compounds relative to cocaine was then calculated.

EXPERIMENTAL

Male Swiss-Webster albino mice were used. After trimming the eyelashes, one drop of the solution to be tested was placed on the surface of each eye. (Grasping the skin near the back of the head caused the eyes to protrude so that the drop readily adhered to the surface.) Approximately 25–30 seconds later, the remaining solution was removed by touching the eyes with a piece of absorbent tissue (Kleenex). One minute from the time the solution was applied, the surface of the cornea was touched with a small glass probe[1] and the presence or absence of the corneal reflex was recorded. Corneal stimulation was repeated at one-minute intervals until the animal failed to show anesthesia on three successive occasions. In testing for the corneal reflex, the animal was placed on a piece of wire mesh and held by the tail with one hand while the other hand was braced on the table top and used to manipulate the glass probe so that the surface of the eye could be gently touched without touching the eyelids. For purposes of calculation, only the first 10-minute interval was used. Thus, each animal contributed 20 responses to the total. The number of times the animal failed to blink was divided by the number of times the eyes were stimulated and the percentage response for each group was plotted against the dose (in mg./ml.). Six to nine mice were used to locate each point on the dose response curve, which was calculated by the method of Litchfield and Wilcoxon (5).

Relative potency was determined by comparing the dose-response curves of each drug to that of cocaine (Table I) and the following results obtained: (*a*) Dyclonine was significantly more active than cocaine. The potency ratio and 19/20 confidence limits were 9.07 and 3.73–21.94. (*b*) Benzonatate did not differ significantly from cocaine. The potency ratio and 19/20 confidence limits were 1.00 and 0.45–2.24. (*c*) Procaine was significantly less active than cocaine. The potency ratio and 19/20 confidence limits were 0.07 and 0.03–0.16.

[1] A piece of glass tubing (0.5 cm. i.d.) was heated and drawn out so that the tip, when sealed, was approximately 1 mm. in diameter. To facilitate the application of this tip to the cornea, the final 2 mm. were bent at a 60° angle and a 30° angle was made 2 cm. above this point. The overall length was about 13 cm.

DISCUSSION AND CONCLUSIONS

These data suggest that the procedure presented may be used for quantitative evaluation of surface anesthetics in mice. Accurate dose-response curves were economically and easily obtained. A further advantage was the fact that stimulation of the unanesthetized cornea of the albino mouse never failed to elicit a blink in our studies to date.

This test, as most tests, is not entirely objective. At the time the drug effect begins to wane, some difficulty may arise in scoring corneal responses. The response which occurs when the unanesthetized eye is touched is clean, clear-cut, and distinct. The total lack of response of the fully anesthetized eye likewise presents no difficulty to the observer. However, at some point between these extremes, the animal squints or blinks in response to corneal stimulation in a manner very different from the normal rapid response. Any movement of the eyelid was taken as lack of anesthetic effect. This was actually a minor problem in the experiment and should not be unduly emphasized.

Since it is common practice to modify procedures, the following variables might well be investigated to improve on the procedure presented. These include: (*a*) Choice of different time intervals. (*b*) Alterations of the time of drug contact with the eye. (*c*) Examination to determine whether there is less variability between the right eye and the left eye than there is between different animals. If so, a more valid comparison of relative potency could be obtained by treating one eye with a standard drug and the other eye with the drug whose relative potency is to be determined. In this manner, both drugs could be compared in the same animal at the same time.

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Isolation of Podophyllotoxin from *Callitrus drummondii*

By LEMONT B. KIER, DOROTHEA B. FITZGERALD†, and SHIRLEY BURGETT

Aqueous suspensions and extracts of the dried needles of *Callitrus drummondii* have been shown to be tumor inhibiting against Sarcoma 37 in mice. Hydrolysis of the water extract has given a good yield of podophyllotoxin which is a known tumor-inhibiting substance.

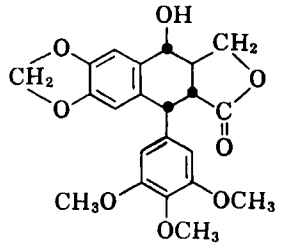
CALLITRUS DRUMMONDII (Parlat) F. Muell is an evergreen tree native to Australia where it is commonly known as Drummond's cypress pine. In a recent survey of conifers for tumor-damaging substances (1), the needles of *C. drummondii* were found to cause necrosis and hemmorrhage in Sarcoma 37 in mice. This study was initiated in an effort to isolate and characterize the principle responsible for this activity.

An initial test indicated that an aqueous suspension of the needles ground to a 100 mesh fineness was active at a level of 100 mg./Kg. against Sarcoma 37 in mice. Extracts of the needles made with ethyl acetate and ethanol were inactive in the same test at levels of 400 mg./Kg. and 750 mg./Kg., respectively. These results indicated that the active principle was highly water soluble, exhibiting a low solubility in nonpolar solvents. A preliminary extraction with water followed by lyophylization produced a residue with an activity of 50 mg./Kg., confirming the belief that the active principle was highly water soluble. Furthermore, the finding of some activity in a xylene defatting solution and the extent depending upon the time of exposure indicated that the active principle could be a glycoside. Partial hydrolysis during processing might well produce a xylene soluble aglycone.

Large-scale defatting and extraction with water followed by lyophylization produced an amorphous hygroscopic material.

Column chromatography of this material was attempted on several adsorbents, silica gel (2) proving to be the most satisfactory. Water eluents became increasingly more active as they became lighter in color. A maximum activity of 2 mg./Kg. was obtained for the light-colored lyophylized residue of the final eluents.

The ultraviolet adsorption of the active fraction showed a high intensity maximum at 290 mμ and a minimum at 260 mμ. The infrared spectrum indicated a very close resemblance to that of podophyllotoxin (I) from an authentic specimen.



(I)

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Hydrolysis of the combined active eluents with emulsin produced glucose, identified as the phenylhydrazone. The aglycone portion was recrystallized from benzene, m.p. 110–113°. This is close to the melting point reported for the hydrated form of podophyllotoxin (I) (3). Drying this hydrate gave a material (m.p. 180–182°) which gave no depression with authentic podophyllotoxin (I). The infrared and ultraviolet spectra were identical.

The finding of podophyllotoxin (I) in a conifer is not unusual. It has been reported in several *Juniperus* species (4, 5). The minimum effective dose for podophyllotoxin (I) against Sarcoma 37 in mice has been reported to be 2 mg./Kg. (4). This activity is consistent with the finding of the activity of the chromatographed fractions of *C. drummondii*. Although this fact does not preclude the possibility that activity may also be due to some other constituent present, it cannot be doubted that podophyllotoxin (I) is certainly largely responsible for the antitumor activity of *C. drummondii*.

EXPERIMENTAL

Plant Material.—The needles were collected in the fall of 1960 near Ravensthorpe, Western Australia, and dried prior to shipment. They were identified as *C. drummondii* (Parlat) F. Muell by T. C. Dunne, Director of Agriculture, Western Australia.

Preliminary Studies.—A quantity of needles were ground to 100 mesh fineness and subjected to antitumor screening.[1] The minimum effective dose of an aqueous suspension was found to be 100 mg./Kg. against Sarcoma 37 in mice. A second portion of ground needles was extracted with water, lyophylized, and the residue subjected to the same test. The M.E.D. was found to be 50 mg./Kg. Extracts of the ground needles with ethanol and ethyl acetate gave no activity at levels of 750 and 400 mg./Kg., respectively.

Extraction.—One-hundred grams of the ground needles were stirred for 5 minutes in 500 ml. of xylene then filtered and dried. The dried marc was vigorously stirred successively in four 1-L. portions of water. The combined aqueous filtrates were lyophylized to a light brown hygroscopic residue weighing 20 Gm.

Isolation of Podophyllotoxin (I) Glycoside.—The lyophylized residue, about 20 Gm., was placed on 1500 Gm. of hydrated silica gel. The column was eluted with water. Fractions were taken according to the intensity of color. The first fractions were dark in color and showed little tumor-damaging activity. As the eluents became lighter in color, the activity rose. The lyophylized residue from the final cuts yielded a light, fluffy, slightly yellow material having a M.E.D. of about 2 mg./Kg.

[1] All antitumor testing was conducted under the auspices of the Cancer Chemotherapy Section, National Institutes of Health, Bethesda, Md.

The ultraviolet absorption of the more active fractions showed a high intensity maximum at 290 mμ and a minimum at 260 mμ. The infrared absorption showed a broad hydroxyl band (3450 cm.$^{-1}$), a lactone (1740 cm.$^{-1}$), and an overall resemblance to a spectrum of podophyllotoxin (I).

Isolation of Podophyllotoxin(I).—The active lyophylized fractions were dissolved in water at pH 5, treated with half their weight of emulsin and allowed to stand 24 hours at 37°. The solutions were then extracted with chloroform in a continuous extractor. The chloroform was concentrated to dryness and the residue recrystallized from benzene, m.p. 110–113°. This melting point is in fair agreement with the value reported for podophyllotoxin (I) benzene hydrate (6, 7). The material was dried for 24 hours *in vacuo* at 100°, m.p. 180–182°. A mixed melting point with authentic podophyllotoxin (I) gave no depression and the infrared and ultraviolet spectra were identical.

Anal.—Calcd. for $C_{22}H_{22}O_8$: C, 63.75; H, 5.35; OCH_3, 22.47. Found: C, 63.88; H, 5.62; OCH_3, 22.18.

The yield of podophyllotoxin (I) from the dried needles based on spectrophotometric analysis was about 1.4 per cent.

The water solution from the chloroform extraction was treated with phenylhydrazone hydrochloride and sodium acetate. The resulting crystals of the phenylhydrazone gave no depression with the corresponding glucose derivative.

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Synthesis and Antifungal Activity of Anilides of Salicylic Acid and *o*-Coumaric Acid

By H. WAYNE SCHULTZ

A series of *o*-hydroxycinnamanilides and the corresponding series of salicylanilide derivatives were prepared and tested for antifungal activity. These compounds were investigated for the purpose of determining the effect of vinylogy and also the effect of substitution on the anilide rings. The results showed that all of the *o*-hydroxycinnamanilide derivatives had less antifungal activity than did their corresponding salicylanilide derivatives. Most of the compounds had some antifungal activity at the tested concentration of 0.5%; however, only two compounds had activity greater than salicylanilide. These compounds were the 3′-chloro- and the 4′-chloro-salicylanilides.

ALTHOUGH a large number of salicylanilide derivatives have been investigated for antifungal activity (1–10), the structural modifications have been generally limited to substitution on one or both of the aromatic rings. It was of interest to note that none of the reported investigations have been concerned with the vinylog derivatives, which are represented by the anilides of *o*-coumaric acid (*o*-hydroxycinnamanilides). Because of the principle of vinylogy (11), it appeared that such derivatives might possess activity similar to that of the related salicylanilide derivatives.

In this investigation a series of *o*-hydroxycinnamanilide derivatives and the corresponding series of salicylanilide derivatives were prepared and tested for antifungal activity. The compounds consisted of the free acids, the unsubstituted anilides, the 2′-, 3′-, and 4′-chloro-anilides, the 2′-, 3′-, and 4′-nitro-anilides and the 2′-, 3′-, and 4′-methyl-anilides. These derivatives not only provided the possibility of determining the effect of vinylogy but also the effect of substitution in varying positions on the anilide rings.

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The author acknowledges with appreciation the cooperation of Dr. K. S. Pilcher in carrying out the antifungal tests.

EXPERIMENTAL

Synthesis of *o*-Hydroxycinnamanilides

***o*-Coumaric Acid.**—From 32.1 Gm. (0.22 mole) of coumarin treated with 400 ml. of 8% sodium hydroxide and 4 Gm. of yellow mercuric oxide according to the procedure of Seshadri and Rao (12), there was obtained 27.5 Gm. (76%) of coumaric acid having a m.p. of 208°. This material was used in the following reaction.

***o*-Acetoxycinnamic Acid (13).**—A mixture of 27.5 Gm. (0.17 mole) of *o*-coumaric acid, 7.0 Gm. (0.85 mole) of sodium acetate and 90 ml. (0.95 mole) of acetic anhydride was heated on a steam bath for 7 hours. After cooling to room temperature, the reaction mixture was poured into 1 liter of ice and water and allowed to stand overnight. The white crystalline material was separated and washed with cold water and dried in air to give 33.0 Gm. of product (94% yield). A sample after recrystallization from benzene gave m.p. 153–154°.

***o*-Acetoxycinnamoyl Chloride (14).**—A mixture of 10.3 Gm. (0.05 mole) of *o*-acetoxycinnamic acid, 11.8 Gm. (0.1 mole) of thionyl chloride and 15 ml. benzene was heated under reflux for $^1/_2$ hour and cooled to room temperature. The solvent and excess thionyl chloride was removed *in vacuo* with gentle heating. Upon standing, a white crystalline material resulted.

4′-Methyl-2-Hydroxycinnamanilide.—In a typical example, the above *o*-acetoxycinnamoyl chloride

The ultraviolet absorption of the more active fractions showed a high intensity maximum at 290 mμ and a minimum at 260 mμ. The infrared absorption showed a broad hydroxyl band (3450 cm.$^{-1}$), a lactone (1740 cm.$^{-1}$), and an overall resemblance to a spectrum of podophyllotoxin (I).

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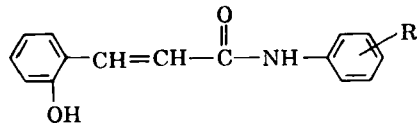
***o*-Coumaric Acid.**—From 32.1 Gm. (0.22 mole) of coumarin treated with 400 ml. of 8% sodium hydroxide and 4 Gm. of yellow mercuric oxide according to the procedure of Seshadri and Rao (12), there was obtained 27.5 Gm. (76%) of coumaric acid having a m.p. of 208°. This material was used in the following reaction.

***o*-Acetoxycinnamic Acid (13).**—A mixture of 27.5 Gm. (0.17 mole) of *o*-coumaric acid, 7.0 Gm. (0.85 mole) of sodium acetate and 90 ml. (0.95 mole) of acetic anhydride was heated on a steam bath for 7 hours. After cooling to room temperature, the reaction mixture was poured into 1 liter of ice and water and allowed to stand overnight. The white crystalline material was separated and washed with cold water and dried in air to give 33.0 Gm. of product (94% yield). A sample after recrystallization from benzene gave m.p. 153–154°.

***o*-Acetoxycinnamoyl Chloride (14).**—A mixture of 10.3 Gm. (0.05 mole) of *o*-acetoxycinnamic acid, 11.8 Gm. (0.1 mole) of thionyl chloride and 15 ml. benzene was heated under reflux for $^1/_2$ hour and cooled to room temperature. The solvent and excess thionyl chloride was removed *in vacuo* with gentle heating. Upon standing, a white crystalline material resulted.

4′-Methyl-2-Hydroxycinnamanilide.—In a typical example, the above *o*-acetoxycinnamoyl chloride

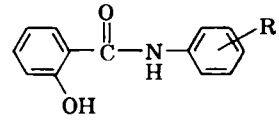
TABLE I.—*o*-HYDROXYCINNAMANILIDES



R	Formula	M.p.,[a] °C.	Yield,[b] %	Carbon,[c] % Calcd.	Carbon,[c] % Found	Hydrogen,[c] % Calcd.	Hydrogen,[c] % Found	Recryst. Solvent
H	$C_{15}H_{13}NO_2$	186–187	69	75.31	74.87	5.44	5.40	EtOH-H_2O
2′-Cl	$C_{15}H_{12}NClO_2$	174–175	49	65.81	66.02	4.39	4.16	EtOH-H_2O
3′-Cl	$C_{15}H_{12}NClO_2$	187–188	55	65.81	65.86	4.39	4.47	EtOH-H_2O
4′-Cl	$C_{15}H_{12}NClO_2$	235–236	63	65.81	65.52	4.39	4.31	EtOH-H_2O
2′-NO_2	$C_{15}H_{12}N_2O_4$	211–212	40	63.39	63.02	4.23	4.12	EtOH-H_2O
3′-NO_2	$C_{15}H_{12}N_2O_4$	237–238	43	63.39	63.78	4.23	4.31	EtOH-H_2O
4′-NO_2	$C_{15}H_{12}N_2O_4$	245–246	61	63.39	63.20	4.23	4.09	Pyridine-H_2O
2′-CH_3	$C_{16}H_{15}NO_2$	171–172	78	75.90	75.42	5.93	6.01	EtOH-H_2O
3′-CH_3	$C_{16}H_{15}NO_2$	170–171	57	75.90	75.51	5.93	5.92	EtOH-H_2O
4′-CH_3	$C_{16}H_{15}NO_2$	223–224	60	75.90	75.59	5.93	6.10	EtOH-H_2O

[a] Melting points are uncorrected and were obtained in capillary tubes. [b] Yield based on *o*-hydroxycinnamic acid. [c] Analyses by Weiler and Strauss, Oxford, Eng.

TABLE II.—SALICYLANILIDES



R	Formula	M.p.,[a] °C.	Yield,[b] %	Carbon,[c] % Calcd.	Carbon,[c] % Found	Hydrogen,[c] % Calcd.	Hydrogen,[c] % Found	Recryst. Solvent
H	$C_{13}H_{11}NO_2$	136–137[d]	79	73.24	73.19	5.16	5.10	EtOH-H_2O
2′-Cl	$C_{13}H_{10}ClNO_2$	165–166[e]	71	63.03	63.20	4.04	4.25	EtOH-H_2O
3′-Cl	$C_{13}H_{10}ClNO_2$	171–172[f]	78	63.03	62.90	4.04	4.11	EtOH-H_2O
4′-Cl	$C_{13}H_{10}ClNO_2$	167–168[g]	83	63.03	63.27	4.04	4.13	EtOH-H_2O
2′-NO_2	$C_{13}H_{10}N_2O_4$	155–157[h]	70	60.47	60.34	3.87	3.75	EtOH-H_2O
3′-NO_2	$C_{13}H_{10}N_2O_4$	216–217[i]	75	60.47	60.29	3.87	3.79	EtOH-H_2O
4′-NO_2	$C_{13}H_{10}N_2O_4$	228–229[j]	77	60.47	60.23	3.87	3.70	MeOH-H_2O
2′-CH_3	$C_{14}H_{13}NO_2$	142–143[k]	62	74.01	74.07	5.73	5.77	Benzene-Ligroin
3′-CH_3	$C_{14}H_{13}NO_2$	135–136[l]	79	74.01	73.93	5.73	5.71	EtOH-H_2O
4′-CH_3	$C_{14}H_{13}NO_2$	155–156[m]	75	74.01	74.11	5.73	5.81	EtOH-H_2O

[a] Melting points are uncorrected and were obtained in capillary tubes. [b] Yield based on salicylic acid. [c] Analyses by Weiler and Strauss, Oxford, Eng. [d] Reported m.p. 136–138° (16). [e] Reported m.p. 168° (17); 167° (10). [f] Reported m.p. 172° (17). [g] Reported m.p. 168–169° (18); 167–168° (4). [h] Reported m.p. 155° (19); 154° (20). [i] Reported m.p. 219° (19); 217–218° (20). [j] Reported m.p. 231° (21); 229–230° (20). [k] Reported m.p. 145° (19); 143–144° (22). [l] Reported m.p. 135–136° (19). [m] Reported m.p. 155–156° (20); 153–156° (23).

(11.2 Gm., 0.05 mole) was dissolved in 25 ml. benzene and a solution of 10.7 Gm. (0.1 mole) of *p*-toluidine in 25 ml. benzene was added slowly with stirring. The resultant mixture was refluxed for 15 minutes on a steam bath and then placed in an evaporating dish and allowed to evaporate to dryness. The solid residue was finely powdered, mixed with 300 ml. of 3% hydrochloric acid, and allowed to stand overnight. The solid material was separated with filtration and washed well with water. This material was dissolved in 150 ml. of 0.1 *N* sodium hydroxide solution and refluxed for 2 hours. After cooling to room temperature, the solution was acidified with hydrochloric acid and diluted with 200 ml. of water. The solid was collected, washed with water, and recrystallized from aqueous ethanol to give 8.1 Gm. of light tan crystals, m.p. 223–234°.

Synthesis of Salicylanilides

2′-Chlorosalicylanilide.—In a typical example a mixture of 10.4 Gm. (0.075 mole) of salicylic acid, 17.7 Gm. (0.15 mole) thionyl chloride, and 15 ml. benzene was refluxed for ½ hour and then cooled to room temperature. The solvent and excess thionyl chloride were removed *in vacuo* with gentle heating. The product was dissolved in 25 ml. benzene and a solution of 19.1 Gm. (0.15 mole) of *o*-chloroaniline in 25 ml. benzene was added slowly with stirring. The resultant mixture was refluxed for 15 minutes on a steam bath and then placed in an evaporating dish and allowed to evaporate to dryness. The solid residue was finely powdered, mixed with 300 ml. of 3% hydrochloric acid, and allowed to stand overnight. The solid material was separated with filtration and washed well with water. Upon recrystallization from aqueous ethanol there was obtained 13.2 Gm. (71%) of white crystals, m.p. 165–166°.

Antifungal Activity

The compounds were tested for their ability to inhibit the growth of three pathogenic species of fungi using a procedure similar to that described by Kligman and Rosenweig (15). Petri dishes containing sterile Sabouraud's agar were innoculated with the test organisms and a sterile filter paper disk was placed in the center of each dish. Test

TABLE III.—MINIMUM WIDTH[a] OF ZONES OF INHIBITION, IN MILLIMETERS, OF 0.5% SOLUTIONS

	T. mentagrophytes	*T. rubrum*	*E. floccosum*
2-Hydroxycinnamanilide	14	25	19
Salicylanilide	39	61	53
2′-Chloro-2-hydroxycinnamanilide	17	41	23
2′-Chlorosalicylanilide	20	51	33
3′-Chloro-2-hydroxycinnamanilide	21	36	25
3′-Chlorosalicylanilide	22	71	48
4′-Chloro-2-hydroxycinnamanilide	0	0	0
4′-Chlorosalicylanilide	35	76	61
2′-Nitro-2-hydroxycinnamanilide	0	0	18
2′-Nitrosalicylanilide	13	24	16
3′-Nitro-2-hydroxycinnamanilide	0	0	0
3′-Nitrosalicylanilide	13	34	26
4′-Nitro-2-hydroxycinnamanilide	0	0	16
4′-Nitrosalicylanilide	21	60	38
2′-Methyl-2-hydroxycinnamanilide	16	24	22
2′-Methylsalicylanilide	23	54	32
3′-Methyl-2-hydroxycinnamanilide	26	33	30
3′-Methylsalicylanilide	27	57	37
4′-Methyl-2-hydroxycinnamanilide	14	0	0
4′-Methylsalicylanilide	17	46	35
2-Hydroxycinnamic acid	0	0	0
Salicylic acid	0	0	14

[a] Average of two measurements.

solutions of the compounds at 0.5% concentration were prepared using alcohol or acetone as the solvent. Alcohol (95% w/v) was used for all of the compounds except the 2′-, 3′-, and 4′-nitro-2-hydroxycinnamanilide derivatives and the 3′- and 4′-nitrosalicylanilide derivatives. For these compounds, which were insoluble in alcohol at the desired concentration, acetone was used as the solvent. To each filter paper disk, 0.8 ml. of a test solution was applied by means of a pipet. Two plates of an organism were treated with each test solution. The pure solvents, alcohol and acetone, were also tested. The plates were incubated at 28° for 72 hours. At the end of this time the diameter of the zone of inhibition was measured.

DISCUSSION

As shown in Table III, all of the *o*-hydroxycinnamanilide derivatives had less antifungal activity than did their corresponding salicylanilide derivatives. Most of the compounds had some antifungal activity at the tested concentration of 0.5%; however, only two compounds had activity greater than salicylanilide. These compounds were the 3′-chloro- and the 4′-chloro-salicylanilides. Of these two, the 4′-chlorosalicylanilide derivative had greater activity against *T. rubrum* and *E. floccosum* than salicylanilide and had an activity against *T. mentagrophytes* which was almost equal to that of salicylanilide.

Among the substituted chloro- and nitro- salicylanilide derivatives it was found that the 4′- position gave the greatest antifungal activity, while the 3′-position was the most effective of the methyl derivatives. The anilide derivatives of *o*-hydroxycinnamic acid did not show this overall consistency in relationship between position and activity. The results further showed that the activity of salicylanilide was enhanced only by the substitution of a 4′-chloro group on the anilide ring, while with its vinylog, *o*-hydroxycinnamanilide, substitution of either 2′-chloro, 3′-chloro, 2′-methyl, or 3′-methyl enhanced its activity.

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Synthesis of a Vinylog of *p*-Aminobenzoylglutamic Acid

By C. DEWITT BLANTON, JR., and W. LEWIS NOBLES

In an attempt to prepare a folic acid vinylog, a vinylog of *p*-nitrobenzoylglutamic acid has been prepared using an alkyl carbonic-carboxylic acid-mixed anhydride. This intermediate nitro vinylog was reduced in the presence of Raney nickel to give a vinylog of *p*-aminobenzoylglutamic acid.

IN PURSUING a program dealing with the preparation of a vinylog of folic acid (pteroylglutamic acid), a relatively large quantity of *p*-nitrocinnamoylglutamic acid and *p*-aminocinnamoylglutamic acid was desired. Since the method (1) reported in the literature for preparing *p*-nitrobenzoylglutamic acid involved the condensation of *p*-nitrobenzoyl chloride and glutamic acid, it was believed that the condensation of *p*-nitrocinnamoyl chloride and glutamic acid would give the vinylog corresponding to *p*-nitrobenzoylglutamic acid. The preparation of *p*-nitrocinnamoylglutamic acid by this technique has been reported (2) to give a 37% yield of a pale yellow material melting at 206–208°. The modified Schotten-Baumann described by Siegart and Day (3) for the preparation of *p*-nitrobenzoylglutamic acid when applied to this study failed to improve the yields. In both procedures, *p*-nitrocinnamic acid and glutamic acid can be recovered in considerable quantity. This might have been anticipated due to the much higher degree of reactivity of *p*-nitrocinnamoyl chloride than that of *p*-nitrobenzoylchloride.

At this point, our attention was turned to the use of alkyl carbonic-carboxylic acid-mixed anhydrides for the preparation of amides (4). The use of this method of synthesis has become of increasing importance, especially in the synthesis of peptides (5). This reaction was readily adapted to the synthesis of *p*-nitrocinnamoylglutamic acid.

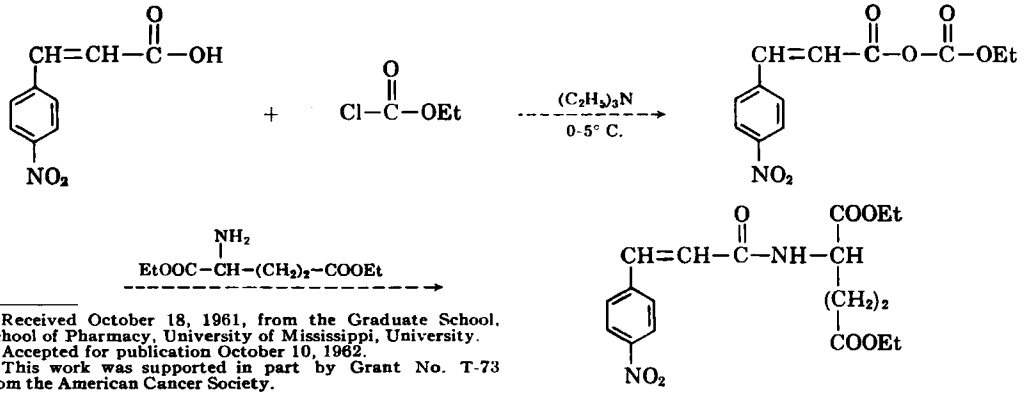
The reaction of the anhydride of *p*-nitrocinnamic acid and ethyl chloroformate with the diethyl ester of glutamic acid proceeded smoothly in diethylene glycol dimethyl ether (diglyme) or tetrahydrofuran (THF) at 0–5°. The diethyl ester of glutamic acid was employed to avoid possible interaction with the carboxylic groups. Hydrolysis of the diethyl *p*-nitrocinnamoylglutamate obtained in 50 to 73% yield gave a pale yellow product identical to that reported by Carrol (2).

The use of a solution of ammonium sulfide (6) for the selective reduction of the nitro group of cinnamic acids appears to be inadequate here since its basic character and the high reaction temperature (85–90°) is sufficient to split the amide linkage. For the conversion of the diethyl *p*-nitrocinnamoylglutamate to diethyl *p*-aminocinnamoylglutamate, the procedure of Blout (7) was found to be effective. In Blout's work, it was demonstrated that Raney nickel does effectively catalyze the hydrogenation of aromatic nitro groups in preference to aliphatic double bonds conjugated with a benzoid ring, and that it was possible to prepare in good yields amino cinnamic acids and esters from the corresponding nitro compound. However, if 2 to 3 Gm. (wet wt.) of Raney nickel were used, as in Blout's original work, an oil was the only product isolated. If, on the other hand, 1 to $1^1/_2$ Gm. (wet wt.) of Raney nickel were employed, a yellow solid substance was obtained; this proved to be diethyl *p*-aminocinnamoylglutamate. In the latter case, the rate of hydrogen absorption was very slow. Adkins (8) has reported that the use of sufficient quantities of Raney nickel can be employed to reduce esters of certain amino acids to amino alcohols. This may, in part, account for the undesirable oil which was isolated when 2 or 3 Gm. (wet wt.) of Raney nickel was employed.

As the similarity in physical and chemical properties of a compound and its vinylog is well known (9), it has become of interest to learn whether or not vinylogs of folic acid will demonstrate similar biological activity. At this time, the only significant observation occurring among these vinylogous substances has to do with one of their physical properties, *viz.*, melting points (Table I).

EXPERIMENTAL

Diethyl *p*-Nitrocinnamoyl-DL-glutamate.—The general method of Sam (10) was utilized with some modification. The procedure of Pandya (11, 12) was employed for preparing *p*-nitrocinnamic acid. Diethyl glutamate was prepared by the method of Chiles and Noyes (13). To a stirred and cooled



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This work was supported in part by Grant No. T-73 from the American Cancer Society.

TABLE I.—COMPARISON OF MELTING POINTS

Parent Compound	M.p., ° C.	Vinyl Compound	M.p., ° C.[a]
p-Nitrobenzoylglutamic acid	112.5 to 113.5	*p*-Nitrocinnamoylglutamic acid	205–207
Diethyl *p*-nitrobenzoylglutamate	96	Diethyl *p*-nitrocinnamoylglutamate	97–99
p-Aminobenzoylglutamic acid	172–173	*p*-Aminocinnamoylglutamic acid	193.8 to 196.6
Diethyl *p*-aminobenzoylglutamate	143–144	Diethyl *p*-aminocinnamoylglutamate	115.5 to 117

[a] Melting points are corrected.

mixture of 20 Gm. (0.103 mole) of *p*-nitrocinnamic acid in 1 L. of diglyme there was gradually added 10.4 Gm. (0.103 mole) of triethylamine. The resulting solution was maintained at 0–5° during the addition of 13 Gm. (0.128 mole) of ethyl chloroformate. After the mixture had been maintained at 0° for 30 minutes there was added 20.0 Gm. (0.146 mole) of diethyl DL-glutamate dissolved in 100 ml. of diglyme; the temperature was maintained at 0 to 5°. The contents of the reaction vessel were allowed to warm to room temperature (32°) and stirred at that temperature for 6 hours. A white crystalline material (triethylamine hydrochloride) was collected by filtration. The yellow solution was allowed to stand overnight at room temperature. Five-hundred milliliters of distilled water was then added to this yellow solution, followed by the addition of chipped ice until precipitation was complete. There was obtained 25.7 Gm. (66.0%) of diethyl *p*-nitrocinnamoyl-DL-glutamate. After recrystallization to analytical purity from an ethanol-water solution, a m.p. of 97–99° was observed.

Anal.—Calcd. for $C_{18}H_{22}N_2O_7$: C, 57.14; H, 5.82; N, 7.14. Found: C, 57.32; H, 5.60; N, 7.46.

Diethyl *p*-Aminocinnamoyl-DL-glutamate.—Ten grams (0.026 mole) of diethyl *p*-nitrocinnamoyl-DL-glutamate was suspended in 50 ml. of absolute ethanol in a pressure bottle and approximately 1 to 1.5 Gm. (wet wt.) of Sponge Nickel Catalyst[1] was added. The bottle was flushed with hydrogen and then shaken mechanically until the rate of hydrogen uptake had decreased tenfold (approx. 24 hours). The catalyst was filtered off and ice-water added to the filtrate. Upon chilling, a pale yellow solid formed. The product obtained in yields of 64–77% melted in the range of 115.5 to 117°.

Anal.—Calcd. for $C_{18}H_{24}N_2O_5$: C, 62.06; H, 6.90; N, 8.05. Found: 62.16; H, 7.12; N, 8.05.

p-Aminocinnamoyl-DL-glutamic Acid.—One gram (0.003 mole) of diethyl *p*-aminocinnamoyl-DL-glutamate was suspended in 25 ml. of 1 *N* NaOH and stirred overnight at room temperature. The resulting solution was chilled and acidified to pH 3–4 with concentrated hydrochloric acid. A cloudy solution resulted but no precipitation occurred. To this solution was added about 1 Gm. of sodium chloride. Upon stirring, a yellow-brown solid appeared. This material was collected on a sintered-glass filter-funnel. Recrystallization from hot water to which a few milliliters of ethanol were added gave a yellow product. This material was observed to melt in the range of 193.8 to 196.6°.

Anal.—Calcd. for $C_{14}H_{16}N_2O_5$: C, 57.53; H, 5.53; N, 9.58. Found: C, 57.54; H, 5.57; N, 9.38.

The presence of a dicarboxylic acid was also confirmed by the determination of its neutralization equivalent.

[1] Division Chemical Co., Cincinnati, Ohio.

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ERRATUM

In the paper titled "Chromatography and Electrophoresis of Phenothiazine Drugs" (1), the last sentence of paragraph two under *Methods* on page 1169 should be changed to read: "The dissolved substances were applied in amounts of 5 to 10 mcg. . . ." Additionally, the last clause of the last sentence of the first full paragraph on page 1170 should be changed to read: ". . . , when concentrations over 10 mcg. per spot were applied."

(1) Mellinger, T. J., and Keeler, C. E., THIS JOURNAL, **51**, 1169(1962).

TABLE I.—COMPARISON OF MELTING POINTS

Parent Compound	M.p., ° C.	Vinyl Compound	M.p., ° C.[a]
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[a] Melting points are corrected.

mixture of 20 Gm. (0.103 mole) of *p*-nitrocinnamic acid in 1 L. of diglyme there was gradually added 10.4 Gm. (0.103 mole) of triethylamine. The resulting solution was maintained at 0–5° during the addition of 13 Gm. (0.128 mole) of ethyl chloroformate. After the mixture had been maintained at 0° for 30 minutes there was added 20.0 Gm. (0.146 mole) of diethyl DL-glutamate dissolved in 100 ml. of diglyme; the temperature was maintained at 0 to 5°. The contents of the reaction vessel were allowed to warm to room temperature (32°) and stirred at that temperature for 6 hours. A white crystalline material (triethylamine hydrochloride) was collected by filtration. The yellow solution was allowed to stand overnight at room temperature. Five-hundred milliliters of distilled water was then added to this yellow solution, followed by the addition of chipped ice until precipitation was complete. There was obtained 25.7 Gm. (66.0%) of diethyl *p*-nitrocinnamoyl-DL-glutamate. After recrystallization to analytical purity from an ethanol-water solution, a m.p. of 97–99° was observed.

Anal.—Calcd. for $C_{18}H_{22}N_2O_7$: C, 57.14; H, 5.82; N, 7.14. Found: C, 57.32; H, 5.60; N, 7.46.

Diethyl *p*-Aminocinnamoyl-DL-glutamate.—Ten grams (0.026 mole) of diethyl *p*-nitrocinnamoyl-DL-glutamate was suspended in 50 ml. of absolute ethanol in a pressure bottle and approximately 1 to 1.5 Gm. (wet wt.) of Sponge Nickel Catalyst[1] was added. The bottle was flushed with hydrogen and then shaken mechanically until the rate of hydrogen uptake had decreased tenfold (approx. 24 hours). The catalyst was filtered off and ice-water added to the filtrate. Upon chilling, a pale yellow solid formed. The product obtained in yields of 64–77% melted in the range of 115.5 to 117°.

Anal.—Calcd. for $C_{18}H_{24}N_2O_5$: C, 62.06; H, 6.90; N, 8.05. Found: 62.16; H, 7.12; N, 8.05.

p-Aminocinnamoyl-DL-glutamic Acid.—One gram (0.003 mole) of diethyl *p*-aminocinnamoyl-DL-glutamate was suspended in 25 ml. of 1 *N* NaOH and stirred overnight at room temperature. The resulting solution was chilled and acidified to pH 3–4 with concentrated hydrochloric acid. A cloudy solution resulted but no precipitation occurred. To this solution was added about 1 Gm. of sodium chloride. Upon stirring, a yellow-brown solid appeared. This material was collected on a sintered-glass filter-funnel. Recrystallization from hot water to which a few milliliters of ethanol were added gave a yellow product. This material was observed to melt in the range of 193.8 to 196.6°.

Anal.—Calcd. for $C_{14}H_{16}N_2O_5$: C, 57.53; H, 5.53; N, 9.58. Found: C, 57.54; H, 5.57; N, 9.38.

The presence of a dicarboxylic acid was also confirmed by the determination of its neutralization equivalent.

[1] Division Chemical Co., Cincinnati, Ohio.

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ERRATUM

In the paper titled "Chromatography and Electrophoresis of Phenothiazine Drugs" (1), the last sentence of paragraph two under *Methods* on page 1169 should be changed to read: "The dissolved substances were applied in amounts of 5 to 10 mcg. . . ." Additionally, the last clause of the last sentence of the first full paragraph on page 1170 should be changed to read: ". . . , when concentrations over 10 mcg. per spot were applied."

(1) Mellinger, T. J., and Keeler, C. E., THIS JOURNAL, **51**, 1169(1962).

Lack of Anticonvulsant Properties of Orally Administered Creatinine in Mice

Sir:

Cade (1) reported that pentylenetetrazol-induced convulsions in guinea pigs were controlled successfully by intraperitoneal injections of creatinine. Further, creatinine injections to control seizures of epileptic patients were encouraging. Pfeifer and co-workers (2) found creatinine administered subcutaneously in rats to show some protection against pentylenetetrazol-induced convulsions in summer but not winter, to abolish convulsions produced by hydration, and to be ineffective against electrically induced convulsions.

We have evaluated creatinine in male albino Swiss-Webster mice, using standard anticonvulsant procedures (3). Mice (groups of ten each) received daily oral administration of creatinine 100, 200, 400, and 800 mg./Kg./day for 4 days. On the third day, the animals were challenged by electroshock (M.E.S. test) 1 hour after the third dose and on the fourth day they were challenged with pentylenetetrazol (Met. test) 1 hour after the fourth dose. No protection was observed. However, deaths following electroshock became progressively less as the dose of creatinine became larger (60, 20, 30, and 0%, respectively). In a second experiment, creatinine was mixed in the diets (0, 0.5, 1.0, and 5.0%) and fed for 7 days to groups of 30 mice per diet. The mice were challenged with electroshock on the third and sixth day, and with pentylenetetrazol on the seventh day. No protection was observed. The number dying was almost identical for the four groups. Weight changes were not appreciably different for the four groups.

We were unable to show anticonvulsant properties for creatinine in mice using two standard anticonvulsant laboratory procedures.

(1) Cade, J. F., *Med. J. Australia*, **2**, 621(1947).
(2) Pfeifer, A. K., Patakyi, I., and Hajdu, P., *Acta Physiol. Acad. Sci. Hung.*, **3**, 153(1952).
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Received March 1, 1963.
Accepted for publication March 26, 1963.

Use of Sulfur Tetrafluoride in Syntheses of Potential Anticancer Agents

Sir:

The recent communication (1) dealing with the synthesis of the trifluoromethyl analog of thymine as a potential anticancer agent has prompted us to report an alternate method of synthesis utilizing sulfur tetrafluoride. Fluoro analogs of many of the naturally occurring pyrimidines have been prepared and tested as potential anticancer agents. Specifically, the trifluoromethyl group has been substituted on the 2, 4, and 6 positions of pyrimidines (2) by classical procedures utilizing a convenient two or four carbon starting material derived from trifluoroacetic acid. For several years we have tried unsuccessfully to prepare 5-trifluoromethyluracil by condensation of ethyl 3,3,3-trifluoropropionate, urea, and ethylorthoformate,[1] a modification of the procedure developed by Whitehead (3).

Since sulfur tetrafluoride was introduced as a reagent for the conversion of the carboxyl group to the trifluoromethyl group (4), many reports have appeared on the selective nature of this reagent (5). Raasch (6) noted the protective effect of excess hydrogen fluoride on the fluorination of aliphatic amino acids. The synthesis of 5-trifluoromethyluracil was successfully completed by a convenient one-step synthesis starting with uracil-5-carboxylic acid. Introduction of fluorine at the 5-carboxyl group in preference to attack of the other reactive sites was observed. The selective nature of the reagent under controlled temperature and with excess hydrofluoric acid (generated *in situ* from water and SF_4) demonstrates the versatility and the many applications possible in the synthesis of trifluoro-

[1] Appreciation is expressed to Donald A. Thompson for exhausting methods of synthesis of 5-trifluoromethyluracil by this approach.

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methyl substituted heterocycles containing oxygen.

Uracil-5-carboxylic acid[2] (1.00 Gm., 0.0064 mole) and 0.5 ml. of water (0.028 mole) were placed in a steel bomb which was sealed. After cooling the bomb in dry ice-acetone, approximately 45 Gm. of sulfur tetrafluoride[3] (0.41 mole) was introduced. This was heated to 100°, agitated for 16 hours and subsequently allowed to cool to room temperature. The volatile material was vented and decomposed in 10% potassium hydroxide solution and the residue was recrystallized several times from water and sublimed giving 0.883 Gm. (77%, m.p. 247–249° dec., reported (1) m.p. 239–241°).

Anal.[4]—Calcd. for $C_5H_3F_3N_2O_2$: C, 33.35; H, 1.68; F, 31.65; N, 15.55. Found: C, 33.49; H, 1.61; F, 31.87; N, 15.69.

The following ultraviolet absorption spectra were recorded: 0.1 *N* HCl, λmax. 257 mμ, ε molar 8150; pH 7, λmax. 257 mμ ε molar 7210; pH 12.2, λmax. 279 mμ, ε molar 6500.

The pharmacological results and the synthesis of this and other analogs will be published in full detail in the future.

(1) Heidelberger, C., Parsons, D., and Remy, D. C., *J. Am. Chem. Soc.*, **84**, 3597(1962).
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Synthesis of Dehydrocycloheximide

Sir:

In addition to our interest in the stereochemistry of cycloheximide (1, 2), we have been concerned with the development of a method for the synthesis of cycloheximide and certain of its degradation products. Recently, Lawes has described the synthesis of anhydrocycloheximide (3); the present communication describes the total synthesis of dehydrocycloheximide.

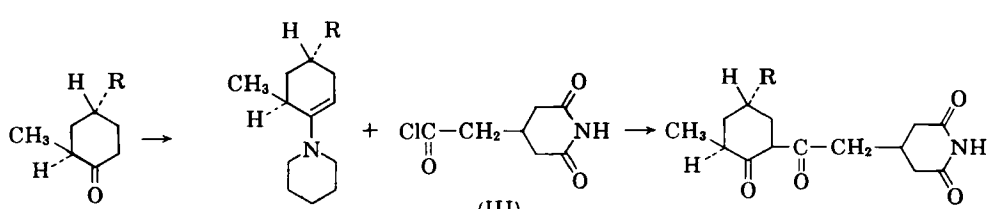
The method which we selected for the synthesis of dehydrocycloheximide employs the combination of the two fragments, 2,4-dimethylcyclohexanone and glutarimide-β-acetyl chloride. The preparation of (+)-*trans*-2,4-dimethylcyclohexanone (I) was accomplished by thermal degradation of cycloheximide (4). When I was allowed to react with piperidine in benzene solution according to the general procedure of Stork (5), a 47% yield of the enamine (II) was obtained; b.p. 79–82°/2.0 mm., $[\alpha]_D^{26} = +44.5°$ (c = 5.66% in ethanol).

Anal.—Calcd. for $C_{13}H_{23}N$: C, 80.76; H, 11.99; N, 7.24. Found: C, 80.58; H, 12.02; N, 7.16.

Condensation of the enamine (II) with glutarimide-β-acetyl chloride (III) (6) in dioxane solution gave, after hydrolysis, a 19% yield of dehydrocycloheximide (IV), m.p. 177–179°, $[\alpha]_D^{20} = -30.9°$ (c = 1.00% in $CHCl_3$).

Anal.—Calcd. for $C_{15}H_{21}NO_4$: C, 64.49; H, 7.58; N, 5.02. Found: C, 64.30; H, 7.37; N, 5.22.

Comparison of the optical rotation, the ultraviolet and infrared spectra, the R_f on thin-layer



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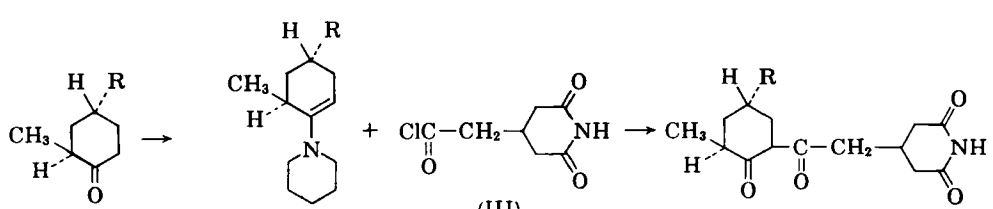

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chromatography, and the melting and mixture melting points of the synthetic product (IV) with an authentic sample of dehydrocycloheximide (7) revealed that the two products were, in fact, identical.

Our research on the synthesis of IV was initiated on the model enamine (VI) prepared from piperidine and 2-methylcyclohexanone (V) since it has been suggested that enamines which are substituted in the 2-position might not undergo reaction (5). However, we have found that enamines of this type are reactive; thus, condensation of the model enamine (VI) with glutarimide-β-acetyl chloride (III) resulted in the formation of a 25% yield of (±)-nordehydrocycloheximide (VII), thereby establishing that acylation at the 6-position is feasible. The reason that the yield of VII and of IV was not over 50% is most probably caused by the fact that the distilled enamine is actually a mixture of double bond isomers in which the double bond is either at C_1-C_6 or C_1-C_2. Since only that isomer in which the double bond is at C_1-C_6 (II or VI) can give IV or VII, the reduction in yield can readily be understood. Evidence that the enamine is a mixture of double bond isomers is available from its infrared spectrum. Thus, in the C=C region, II exhibits two peaks, one at 1680 cm.$^{-1}$ and one at 1645 cm.$^{-1}$ The enamine VI also exhibits peaks at 1670 cm.$^{-1}$ and 1640 cm.$^{-1}$ However, the enamine prepared from cyclohexanone and piperidine exhibits only one peak in the C=C region at 1645 cm.$^{-1}$ This result would be predicted for this enamine since it cannot exist in an isomeric form as can II or VI.

One final point which requires comment is the stereochemical assignment of the methyl groups of dehydrocycloheximide (IV). It has been established that in cycloheximide the methyl groups are *trans* (1, 4), but no proof of the stereochemistry of dehydrocycloheximide has been reported. However, the present synthesis of dehydrocycloheximide (IV) establishes that the methyl groups are *trans* since IV was synthesized from (+)-*trans*-2,4-dimethylcyclohexanone (I). It might be argued that the α-methyl group of I could be isomerized during the preparation of the enamine, but this hypothesis was shown to be rather unimportant when it was found that the acid catalyzed hydrolysis of the enamine (II) regenerated a 75% yield of (+)-*trans*-2,4-dimethylcyclohexanone (I). This result therefore offers strong support to the assignment of the *trans* methyl groups in dehydrocycloheximide (IV).

(1) Schaeffer, H. J., and Jain, V. K., THIS JOURNAL, **50**, 1048(1961).
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(3) Lawes, B. C., *J. Am. Chem. Soc.*, **82**, 6413(1960).
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Book Notices

Year Book of Drug Therapy. 1962–1963 Series. Edited by HARRY BECKMAN. Year Book Medical Publishers, Inc., 35 E. Wacker Drive, Chicago 1, Ill., 1962. 648 pp. 13 × 20 cm. Price $8.50.

A new section has been added to this revised volume entitled "Precautions," replacing the section entitled "Critical Evaluation of the Year's New Drugs." As with prior editions, the major presentation is through abstracts covering reports of new therapeutic or prophylatic uses and applications of drugs during the "series year." The abstracts are well written in the concise and meaty form which has become characteristic of this series. In the newly added section, sketches of accumulated experience regarding the toxic actualities and potentialities of drugs in current use will be of invaluable assistance to pharmaceutical and medical personnel.

Protein Metabolism. Edited by F. GROSS. Springer Verlag, Berlin-Wilmersdorf, Heidelberger Platz 3/, West Berlin, Germany, 1962. xi + 521 pp. 14 × 20.5 cm.

The proceedings of an international symposium are reported—the fourth in a series of such symposia sponsored by Ciba, Ltd., Basle. Major topics covered by the participants include: Action of hormones at the cellular level; Factors influencing protein metabolism in the organism; Evaluation and mode of action of anabolic steroids; Protein metabolism in human pathological states; and

chromatography, and the melting and mixture melting points of the synthetic product (IV) with an authentic sample of dehydrocycloheximide (7) revealed that the two products were, in fact, identical.

Our research on the synthesis of IV was initiated on the model enamine (VI) prepared from piperidine and 2-methylcyclohexanone (V) since it has been suggested that enamines which are substituted in the 2-position might not undergo reaction (5). However, we have found that enamines of this type are reactive; thus, condensation of the model enamine (VI) with glutarimide-β-acetyl chloride (III) resulted in the formation of a 25% yield of (±)-nordehydrocycloheximide (VII), thereby establishing that acylation at the 6-position is feasible. The reason that the yield of VII and of IV was not over 50% is most probably caused by the fact that the distilled enamine is actually a mixture of double bond isomers in which the double bond is either at C_1-C_6 or C_1-C_2. Since only that isomer in which the double bond is at C_1-C_6 (II or VI) can give IV or VII, the reduction in yield can readily be understood. Evidence that the enamine is a mixture of double bond isomers is available from its infrared spectrum. Thus, in the C=C region, II exhibits two peaks, one at 1680 cm.$^{-1}$ and one at 1645 cm.$^{-1}$ The enamine VI also exhibits peaks at 1670 cm.$^{-1}$ and 1640 cm.$^{-1}$ However, the enamine prepared from cyclohexanone and piperidine exhibits only one peak in the C=C region at 1645 cm.$^{-1}$ This result would be predicted for this enamine since it cannot exist in an isomeric form as can II or VI.

One final point which requires comment is the stereochemical assignment of the methyl groups of dehydrocycloheximide (IV). It has been established that in cycloheximide the methyl groups are *trans* (1, 4), but no proof of the stereochemistry of dehydrocycloheximide has been reported. However, the present synthesis of dehydrocycloheximide (IV) establishes that the methyl groups are *trans* since IV was synthesized from (+)-*trans*-2,4-dimethylcyclohexanone (I). It might be argued that the α-methyl group of I could be isomerized during the preparation of the enamine, but this hypothesis was shown to be rather unimportant when it was found that the acid catalyzed hydrolysis of the enamine (II) regenerated a 75% yield of (+)-*trans*-2,4-dimethylcyclohexanone (I). This result therefore offers strong support to the assignment of the *trans* methyl groups in dehydrocycloheximide (IV).

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Practical Therapeutics. By H.-J. B. Galbraith, J. Q. Matthias, and R. C. King. Year Book Medical Publishers, Inc., 35 East Wacker Drive, Chicago, Ill., 1962. vii + 446 pp. 14 × 22 cm. Price $7.50.

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Enzymatic Synthesis of DNA. By Arthur Kornberg. John Wiley & Sons, Inc., 440 Park Ave. South, New York 16, N. Y., 1962. ix + 103 pp. 12.5 × 18.5 cm. Price $4.

This represents the fifth published work in the Ciba Lectures in Microbial Biochemistry. Chapter 1 concerns itself with the replication of DNA—structure, enzymatic approach to replication, properties of the enzyme, evidence for base-pairing mechanism of replication, and some of the unsolved questions. Chapter 2 is entitled "De Novo Synthesis of DNA-like Polymers" and concerns itself with the dAT copolymer and the dGdC polymer. Chapter 3 deals with synthesis of HMC deoxynucleoside monophosphate and HMC deoxynucleoside triphosphate. Dr. Kornberg, one of the recipients of the Nobel Prize in Medicine in 1959, has included figures, tables, structures, and literature references to produce a valuable source of information on the synthesis of DNA.

Encyclopedia of Plant Physiology. Vol. XVII Physiology of Movements, Part 2. Edited by W. Ruhland. Springer-Verlag, Berlin-Wilmersdorf, Heidelberger Platz 3/, West Berlin, Germany, 1962. 17 × 25 cm. xii + 1174 pp. Price DM272.

The volume is an extensive discussion and survey of the basic facts of protoplasmic streaming in plant cells and problems relevant to its mechanism. The biological meaning of this phenomenon is not discussed. The contributed sections are printed in the language of the contributor, mainly English or German. An English to German subject index is included as well as an author index. Extensive literature references are provided following each section.

Advances in Enzymology. Vol. 24. Edited by F. F. Nord. Interscience Publishers, 440 Park Ave. South, New York 16, N. Y., 1962. v + 572 pp. 15.5 × 23 cm.

Included in this volume are contributed sections on Aspects of the Biosynthesis of enzymes; Metabolism of spermatozoa; Chemical modifications of proteins and their significance in enzymology; immunochemistry, and related subjects; Structure and function of ribonuclease; Molecular properties and transformations of glycogen phosphorylase in animal tissues, Distribution of enzymes between subcellular fractions in animal tissues; The effects of ionizing radiation on enzymes; Identical and analogous peptide structures in proteins; and Mechanisms related to enzyme catalysis. Subject and author indexes as well as a cumulative index for volumes 1–24 are included.

Pyridine and its Derivatives. Vol. 14, Part III. Edited by Erwin Klingsberg. Interscience Publishers Division of John Wiley & Sons, 440 Park Ave. South, New York 16, N. Y., 1962. ix + 914 pp. 15.5 × 22.5 cm. Price $65.00.

Included in this third part of a four-part work, are Aminopyridines, Pyridinecarboxylic acids, Pyridine side-chain carboxylic acids, and Pyridinols and pyridones. As part of a larger series, "The Chemistry of Heterocyclic Compounds," the editors and authors have made a splendid contribution towards making a subject of such importance and intrinsic difficulty readily available. Extensive literature references carefully document each section to permit ready access to original reports.

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The Editor comments

OPPORTUNITY FOR GROWTH

The A.PH.A. Scientific Section has had a long history of valuable service to the pharmaceutical scientist. While the record of the A.PH.A. Industrial Pharmacy Section is considerably shorter, nevertheless, even during its relatively brief history it has made a very significant contribution to the profession.

In an effort to consider how the needs and desires of their respective members might be served even better, the officers of these Sections have held discussions to explore the possibility of pooling their individual efforts toward the formation of a single, unified group. Much of the original impetus for such a reorganization came from a combined Section resolution which was passed during the 1962 Las Vegas annual meeting and referred to the A.PH.A. Council for study. Recognizing the great potential value of the suggested plan to this vital segment of the ASSOCIATION membership, both the Council and the A.PH.A. staff endorsed the principle of the general proposal and encouraged the officers of the Scientific and Industrial Sections to discuss the plan with their respective Section memberships. In order to meet the expanded services which the membership would require, the employment of a full-time staff person to serve as permanent Secretary of the proposed group was also considered.

After subsequent consideration and preliminary study on the part of the Section officers, a broadly defined organizational structure was drawn up, and the "*Academy of Pharmaceutical Sciences*" was suggested as a name for the group. The Section officers then referred the proposals to their respective memberships in order that the matter might be given further consideration at this year's annual meeting.

During the meeting this May, resolutions were adopted by each Section calling for the establishment of a formal Committee composed of representatives designated by each of the interested Sections. The Committee is to be charged with the responsibility of exploring the feasibility of creating such a unified body of pharmaceutical scientists, and with developing a more detailed proposal for consideration by the individual Section memberships. The action taken this May might well represent the initial step in what eventually could lead to unprecedented growth for the pharmaceutical sciences, for while enormous strides have already been made, the ultimate potential is even far greater. The ability of such an Academy to realize these goals will depend largely upon the continued efforts of the Section officers and memberships in patiently, but resolutely working out a more defined proposal for its establishment.

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Review Article

Pharmaceutical Aerosols

By JOSEPH L. KANIG

In May of 1960 a ten-page survey questionnaire was mailed to approximately one-hundred pharmaceutical companies. The purpose of this survey, instituted by a manufacturer of aerosol containers, was to elicit information regarding the needs of the pharmaceutical industry in its projected thinking on the potential of the pharmaceutical aerosol. The results of this survey (227) indicated a greater need for dissemination of more information on the pressure package throughout the pharmaceutical industry than had been realized. This was apparent from the widely divergent views on aerosol components and disagreement on potential aerosol applications. The survey also concluded that only a small number of companies possessed sufficient experience to make considered statements.

The unreliability of questionnaire surveys is well known. Nevertheless, this survey revealed a large void in the scientific and technical knowledge of the principles of aerosols as they pertain to pharmaceuticals. Considering the extent and volume of prognostication in this area (9, 10, 29, 96, 101, 124, 155–157, 159, 163, 171, 173, 185–191, 196, 241), one may speculate as to the reasons why this void has not yet been filled. They may range from traditional conservatism among members of the pharmaceutical industry to the lack of specially trained and experienced research personnel to conduct the required experimental studien In all likelihood, one of the major reasons has bees. the time required for product development and research personnel to assimilate the new concepts and technologies involved in pressure packaging, and to bring these into play in the design of pressurized dosage forms. Important innovations in medical therapy and dosage forms do not generally spring full grown from idea to practice in short periods of time.

The advantages which are claimed for pharmaceutical aerosols are, for the most part, valid enough to command consideration. Evidence of the unique properties of some of these ideas has already been manifested by several of the products in current use (64, 69, 70, 80, 90).

Lists of pharmaceutical aerosols available in the United States (10, 189) and abroad (4) are beginning to indicate a trend away from products offering only push-button convenience toward those which are truly unique and presumably more effective therapeutically. Advantages of aerosol therapy are being brought to the attention of medical practitioners (136) and this increased familiarity with the properties of aerosol pharmaceuticals is contributing to the upsurge of interest.

Another barometer of the increase in aerosol development is the number of patents issued in this area. Most of these are concerned with valves, containers, and special adapters for use

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with pharmaceuticals. Methods of applying aerosols to the eye (234), for administering medication orally into the lungs (21, 22, 71, 158), pressurized douches (27), and special aerosol formulations for use in veterinary medicine (30, 60, 228) are a few of the many patents which have been issued in recent years.

Most of the advances which have been made in aerosol technologies have been concerned with the development of valves, containers, and propellants. These are the essential components of the pressure system and it is therefore understandable that emphasis should have been placed on their development. It is not the intent of this review to delve into the specifics of these components other than to indicate their role in the problems associated with the development of aerosol pharmaceuticals. The reader is directed to two books (96, 196) which adequately present the basic principles of aerosol technology together with discussions of the factors in formulating and filling aerosols.

PROPELLANTS

One of the most intriguing aspects of aerosol formulation is the dual role played by most of the currently available propellants. These supply the necessary energy required by the self-propelling features of the aerosol, and, in addition, become an integral component of the contents. In many instances the propellant used may be quite satisfactory in supplying pressure, but at the same time introduces serious problems which affect therapeutic efficiency and product stability. The extent to which these problems can be solved will ultimately dictate the range and type of pharmaceutical applications of aerosol systems.

Propellants in current use may be divided into two major categories: liquefied or compressed. The bulk of the liquefied gas propellants are fluorochloro derivatives of methane and ethane. These halogenated propellants are marketed throughout the world under a variety of names, but most of them are also designated by a numbering system which generically indicates the number and identity of atoms in each compound. The newest member of this family of propellants is octafluorocyclobutane (Propellant C-318). This nonchlorinated cyclic compound is considered the most stable of the halocarbon propellants and has been cleared for use with edible products. Its potential in pharmaceutical applications has not yet been fully explored (88). Reed (166) has secured a patent for a mixture of octafluorocyclobutane (Freon C-318) and isobutane which he claims is suitable for pressurized aqueous products which have either an acid or alkaline pH and are intended for cosmetic or pharmaceutical application.

Among the liquefied halocarbon propellants, trichloromonofluoromethane (Propellant 11) is somewhat more reactive than the others and undergoes hydrolysis in the presence of water and metal catalysts. Solutions containing alcohol and Propellant 11 also become acidic and develop unpleasant odors (233). It has been demonstrated by Sanders (178) that this reaction is not due to hydrolysis but rather to a free radical reaction between Propellant 11 and ethyl alcohol. Experiments with several types of free radical inhibitors showed that nitromethane, a colorless, odorless, nontoxic liquid, was suitable for stabilizing solutions against the reaction (26). Propellant 11 stabilized with nitromethane is now commercially available as "Freon 11" S and seems to offer advantages in the formulation of stable alcohol based solutions.

Hydrocarbon propellant gases, such as propane, isobutane, and *n*-butane have enjoyed limited use in nonpharmaceutical aerosols (2, 36). Their chief disadvantage is their flammability when used alone, but it is claimed that this hazard is minimized when these compounds are mixed with the halocarbon propellants. The possibility of leaks of these flammable propellants from stored containers, however, has become a matter of concern to city fire departments (3, 152).

The nonliquefied or compressed gas propellants such as carbon dioxide, nitrous oxide, or nitrogen (93, 104, 131, 164, 226), and chlorinated propellants such as vinyl chloride (193), methylene chloride, or chlorothene have been used in limited capacities (113).

AEROSOL DOSAGE FORMS

The wide range of pressures and properties offered by the available propellant gases together with the variety of valves (both metering and continuous action) and containers make it theoretically possible to design aerosols which possess advantages not normally encountered in conventional dosage forms. For example, anhydrous suspensions of mucolytic enzymes were developed in this laboratory and were found to retain their enzymatic activity when stored under ambient conditions for extended periods of time. This is not easily possible in any other ready-for-use form. The growth of the aerosol as a dosage form depends, to a large degree, on the ability of the pressurized units to offer similar advantages. Since the attainment of unique features is almost always attended with the necessity of solving

unique problems, a review of the basic types of aerosol dosage forms is in order before considering specific applications.

Solutions

Most of the liquefied propellants used in aerosol formulations exhibit solvent characteristics similar to the nonpolar organic solvents. They are very poor solvents for polar compounds and are immiscible with polar liquids. This rather severe limitation inherent in the properties of the propellants has prevented their use alone in those instances where solutions are desirable or required.

The solvent power of the various propellants for different materials has been investigated only to a limited extent (11, 18, 209). Several methods for describing or evaluating the solvent power of the propellants have been developed (100, 149, 151) and used in solubility studies. A qualitative method of estimating miscibility is the use of kauri-butanol values. These are obtained by titrating 20 Gm. of a standardized solution of kauri gum in butyl alcohol at 77° F. with the liquid under consideration. The end point is reached when turbidity produced due to precipitation of the gum prevents reading 10 point type through the solution in a 250-ml. Erlenmeyer flask. This method is used in the solvent and paint industries and seems to agree with results obtained in solubility studies (126). The kauri-butanol values for halocarbon propellants have been reported (210).

Considering the advantages to be gained, it is surprising to find that very little has been done to circumvent the problems imposed by the poor solvent properties of the propellants for polar compounds. The results of work done in this area may be divided into two types of aerosol systems.

Three-phase Systems.—One method (57) of obtaining sprays containing water-soluble compounds is the use of the three-phase system. This aerosol contains water or an aqueous solution plus the liquefied propellant which forms a separate layer, either below or above the aqueous layer, depending on relative liquid densities. The two immiscible liquid layers and the vapor in the headspace of the sealed container constitute the "three phases" of such systems. When the propellant layer is on the bottom of the container, a dip tube, or standpipe, must be attached to the valve and its length adjusted so that it does not extend into the liquid propellant phase (Fig. 1). In such systems, components which are soluble in, or miscible with, the aqueous layer are maintained as a solution until sprayed.

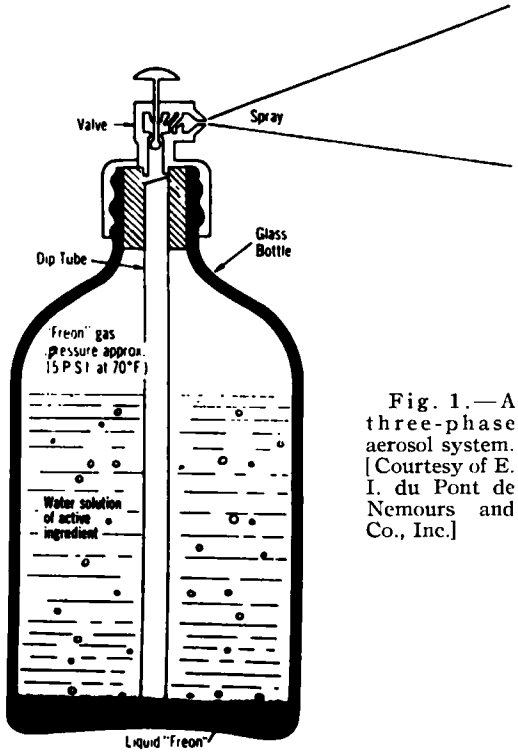

Fig. 1.—A three-phase aerosol system. [Courtesy of E. I. du Pont de Nemours and Co., Inc.]

Actually the propellant phase serves to supply the pressure required to force the aqueous solution up the dip tube and out of the container. It does not materially affect the character of the spray; this becomes the function of the valve components. None of the liquefied propellant is introduced into the aqueous solution while it is being ejected and the atomized spray which results is therefore similar to an aqueous spray produced by conventional squeeze-type atomizers.

Variations of the three-phase type consist of water-based products dispensed by means of the vapor pressure produced by floating small quantities of flammable hydrocarbon gases on top of the aqueous layer. These sprays offer no advantages over those which employ denser halocarbon propellants since the spray characteristics are still limited and pose the additional problem of the possibility of highly flammable liquids being dispensed when the container is nearly empty.

The three-phase system is very similar in operation to the use of compressed gas as the propellant force. A compressed gas (such as nitrogen) occupies the headspace above the liquid solution and simply exerts sufficient pressure on the liquid to force it up the dip tube when the valve is actuated. Here, too, the spray characteristics are limited to the mechanical break-up action of the valve components, and the entire system is sub-

ject to the disadvantages imposed by the use of compressed rather than liquefied gases. Notable among these disadvantages are: (*a*) a large gas space is required in the container to maintain sufficient pressure to empty the contents, (*b*) pressure decreases as the contents are discharged giving rise to changes in the nature of the spray, and (*c*) the possibility of misuse causing an accidental discharge of gas content which produces an inoperable product.

A patented multi-component three-phase system (36) claims to overcome the cited objections and limitations by employing a mixture of propellants, a hydro-alcoholic phase, and a specially designed valving device. The propellant phase is composed of a mixture of halocarbon and hydrocarbon propellants. Hydrocarbon propellants suggested include propane, isobutane, isopentane, or 2,2-dimethyl propane. The mixture of propellants is adjusted so as to possess a liquid density almost equal to but not exceeding the density of the hydro-alcoholic phase. In this manner the propellant phase floats on top of the other, in the static state, but the two are easily mixed by moderate agitation of the container prior to use. The alcohol (ethyl or isopropyl) content helps in dispersing the propellant liquid throughout the aqueous phase. The water content is said to inhibit flammability, reduce cost, and as a component of the hydro-alcoholic phase, makes possible the use of solutes which are soluble in water and alcohol mixtures.

The admixture of the propellants and hydro-alcoholic solution makes it possible to produce sprays with a variety of desirable characteristics as the dispersed propellants rapidly boil away from the spray. This feature is supposedly assisted by the valving arrangement which is comprised of a mixing chamber to premix the liquid and vapor phases before emission from the valve.

Two-phase Systems.—Aerosol terminology defines the two-phase system as having one vapor and one liquid phase. Any nonvolatile material dissolved in the liquid phase is left suspended in air as a fine dispersion immediately after spraying as the liquefied propellants boil rapidly away. Because of the poor solvent power of the halocarbon propellants for polar compounds, only nonpolar materials are directly dissolved by them. Efforts to increase the solvent power of the liquefied halocarbon propellants have centered mainly on the use of co-solvents. Primary objectives of such studies are the development of water-based aerosol formulations which would permit the incorporation of ingredients soluble in water, reduce the flammability hazard, and decrease formulation costs. A successfully formulated two-phase aerosol is also the simplest to manufacture. The added solvent becomes an important factor in such systems. It not only diversifies and enhances the solvent action of the propellant, but also exercises a considerable role in determining the characteristics of the spray produced and the applicability of the solution.

Early formulations which appeared employed alcohol as the co-solvent. A patent issued in 1959 (158) describes the use of ethyl alcohol in providing medicaments in aerosol form suitable for inhalation therapy of water-soluble drugs such as isoproterenol hydrochloride, epinephrine hydrochloride, and phenylephrine hydrochloride, or alcohol-soluble materials such as atropine, octyl nitrite, and ephedrine. The liquid phase of the aerosol, according to this patent, consists of the medicament dissolved in ethanol (20–40%) and water (1.5–2.0%), with the remainder of the composition consisting of liquefied halocarbon propellants designed to produce a total vapor pressure of between 20 and 40 pounds per square inch gauge (psig) at 70° F. Antioxidants such as sodium ascorbate, ascorbic acid, or sodium bisulfite are added as stabilizers where required.

A more recent patent (205) utilizes essentially the same procedure for producing aerosol compositions containing isoproterenol hydrochloride for oral inhalation. This patent, however, claims that the use of ascorbyl palmitate as an antioxidant for such solutions is superior to ascorbic acid since the latter agent comes out of alcohol solution in the presence of halocarbon propellants. Isoproterenol hydrochloride is stated to be more suitable than isoproterenol sulfate for the same reason. Apparently, the physical-chemical laws which govern stability of nonpressurized solutions must not be overlooked in formulating pressurized medicaments.

Additional attempts to produce aqueous two-phase systems successfully have utilized a variety of co-solvents. A rather extensive study of co-solvents has been conducted by Geary (72), who first determined the miscibility of the solvents with propellants and then conducted solubility studies of the ternary systems of water, halocarbon propellants, and co-solvents. The ternary plots of these systems are excellent guides in formulating water-based aerosol products with ketones, alcohols, glycols, and other solvents. A similar study (215) has been conducted with glycols, glycol ethers, and polyglycols as the co-solvents.

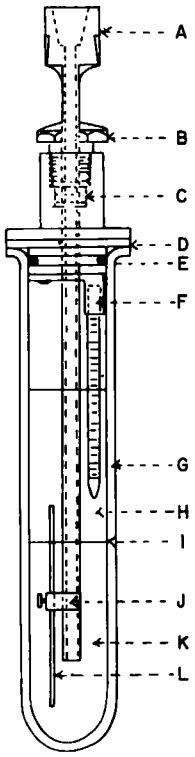

Fig. 2.—A pressure tensiometer for the measurement of surface and interfacial tensions in pressure systems. [From Reference 108 (patent pending).]

On the basis of published data, many of these systems appear to offer improved solvent power for two-phase aerosol formulations together with a wide range of spray characteristics. The major problem associated with their use, however, is the toxicity of many of the solvent materials. Solvents such as acetone, methanol, the "carbitols," and "cellosolves" are almost automatically excluded from pharmaceutical applications in the quantities required. Others, such as 1,3 butylene glycol, dipropylene glycol, and triethylene glycol would require additional toxicity and irritation data before they could be considered for even topical application.

Modification of Interfacial Barrier.—The use of surface-active agents in preparing two-phase aerosol solutions has only recently been considered (106, 108, 110). Surfactants had previously been suggested as a means of forming dispersions with propellants and water. These dispersions were described (32) as being "neither true solutions, nor emulsions, but . . . somewhere in between." These authors proposed the use of nonionic surfactants such as Tweens and Spans, but it was not made clear as to whether water was dispersed in propellants or *vice versa*.

Current concepts and knowledge of the functions of surfactants in solubilizing materials make it obvious that such activity can be successfully applied to problems in aerosol solutions. The relationships which have been established between the physical-chemical properties of surfactants and their specific activities in nonpressurized systems could conceivably be defined for aerosols as well. A study has been reported which was conducted to initiate the development of procedures to measure the effects of surfactants in aerosols (108). The first requirement in this study was an accurate method of measuring the surface tension of the aerosol propellants and the interfacial tension of the propellants with other immiscible liquids. Of the several methods available for the measurement of surface and interfacial tensions, only the capillary height method was found to be adaptable for use with pressure systems. The capillary height method is generally considered to be the most accurate of all, partly because the theory has been developed with considerable exactitude and partly because the experimental variables can be closely controlled (89).

Figure 2 illustrates the pressure tensiometer which was designed to embody the basic principles of the capillary height method in an instrument capable of controlling the unique variables encountered in working with liquefied gases. The capillary tube (*L*) was positioned at the surface or interface of the contents of the glass pressure tube (*G*) by adjusting the height of capillary tube mounting (*J*) or raising or lowering the inlet tube (*A*) after loosening the gland nut (*B*). Escape of pressurized contents was prevented by stainless steel gland washers and a neoprene gland (*C*) plus "O" ring and washers (*E*). Liquefied propellants were introduced into the pressure chamber by means of a needle valve inserted in the coupling on top of the Monel inlet tube (*A*). Water or any other fluid was admitted by means of the same tube but under a positive pressure just exceeding the internal pressure created by the particular propellant in the chamber. A calibrated thermometer inserted into a bracket (*F*) indicated internal temperature.

The capillary height was measured at constant temperature by means of a micrometer slide cathetometer with a 50-mm. range and an accuracy of 0.005 mm. Surface and interfacial tensions were calculated by

$$\gamma = {}^1/_2\, rhg\,(d - d') \qquad \text{(Eq. 1)}$$

where r = radius of the capillary tube, h = height of the meniscus, g = acceleration due to gravity, and $(d - d')$ = difference in density between liquid and vapor phases.

The pressure tensiometer was used to measure surface tensions of selected propellants. The

TABLE I.—SURFACE TENSIONS OF LIQUEFIED HALOCARBON PROPELLANTS

Propellants	Dynes/cm. @ 25°C. Theoretical Value,[a] Parachor Method	Dynes/cm. @ 25°C. Experimental Value, Pressure Tensiometer
Propellant 11 $C Cl_3 F$	19	18.86
Propellant 113 $C Cl_2 F$–$C Cl F_2$	19	18.40
Propellant 114 $C Cl F_2$–$C Cl F_2$	13	11.66
Propellant 12 $C Cl_2 F_2$	9	8.41

[a] Technical Bulletin B-2, E. I. du Pont de Nemours and Co.

TABLE II.—INTERFACIAL PROPERTIES OF WATER PROPELLANT SYSTEMS

Interface	Interfacial Tension, Dynes/cm. @ 25°C. Without Surfactant	With Surfactant[a]
Water/Propellant 11	49.73	12.26
Water/Propellant 113	49.12	6.21
Water/Propellant 12	56.70	3.52
Water/Propellant 114	54.60	2.25

[a] Igepal CO-530, 0.1% w/v, dissolved in propellant phase.

resulting values compared favorably with theoretical values (Table I). Interfacial tensions of propellant-water systems with and without surfactants present were also determined. Table II indicates the effect of one surfactant on these interfacial tensions.

Several interesting and potentially useful preliminary observations were made possible by use of the pressure tensiometer. One was the linear relationship (Fig. 3) between the solvent power of four different halocarbon propellants and the interfacial tension against water with 0.1% of a nonionic surfactant dissolved in the propellants. This introduces the possibility of another parameter in the ability to predict and control the interfacial barrier. Since the liquefied halocarbon propellants are rarely used alone, but in mixtures, the resulting broad range of solvent power for any given surfactant makes possible a corresponding range of interfacial tensions.

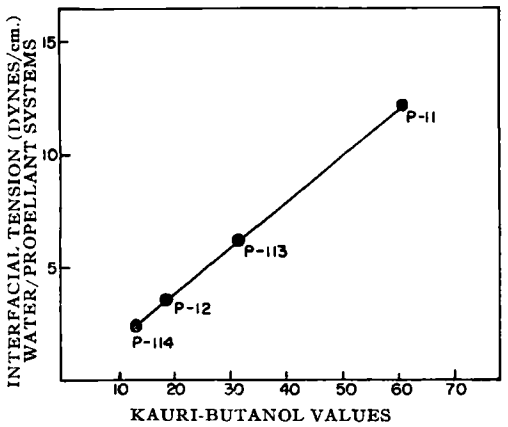

Fig. 3.—Relationship between solvent power of propellants and the interfacial tension against water with 0.1% Igepal CO-530 dissolved in the propellants. [From Reference 108.]

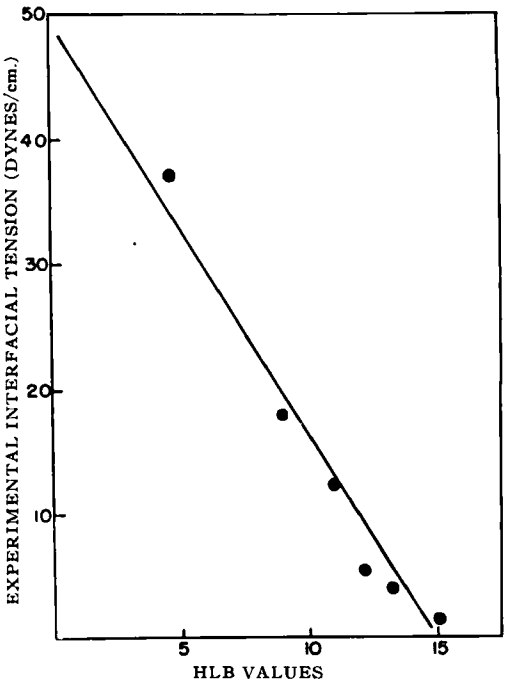

Fig. 4.—Interfacial tension *vs.* known HLB values. [From Reference 108.]

Chun and Martin (35) have demonstrated a linear relationship between interfacial tensions and HLB values of surfactants in toluene-water systems. The pressure tensiometer was employed to establish a similar relationship for surfactants in propellant-water systems. The Igepal CO surfactants were used in Propellant 11-water systems. Interfacial tensions obtained with 0.1% surfactant dissolved in the propellant plotted against HLB values resulted in a linear relationship as shown in Fig. 4. By means of the method of least squares, the equation of this line is given by

$$\gamma = 49.5 - 3.32\,(\text{HLB}) \qquad \text{(Eq. 2)}$$

Utilizing known HLB values for the Igepal CO surfactants, theoretical interfacial tensions in Propellant 11-water systems were calculated by means of Eq. 2. These values and their deviation from experimental values are shown in Table III.* Agreement between calculated and experimental values has encouraged additional studies in this laboratory which currently seek to expand these relationships in the use of surfactants to

* See p. 533 for Table III.

solubilize polar compounds directly in propellant mixtures.

Suspensions

Of all the aerosol dosage forms which have appeared since the time the pressure package became technically feasible, the one offering the greatest challenge in successful formulation is that which consists of a suspension of finely powdered insoluble materials in the liquefied propellants. The ability to dispense a self-propelled dry powder from an hermetically sealed container offers the opportunity for aerosol formulations which are uniquely different from their nonpressurized counterparts.

Attempts to suspend powders in liquefied propellants introduce several problems. Foremost among these is the agglomeration of the suspended particles. This most often occurs immediately after the suspension is prepared, or within a short period thereafter. The rate and extent of agglomeration are apparently modified by the composition of the suspended solids. Agglomeration results in a clogged valve, either initially or after it has been in use for a while. Where uniformity of dosage is imperative, agglomerated particles may cause variation in the amounts of powder delivered even if the valve remains operable. Another problem is the tendency of the suspended powders to become deposited in a cake form on the inner walls of the container, particularly in the area above the liquid level, the headspace. This results in a loss of active ingredients which are not dispensed from the container. Inaccurate dosage also results because the concentration of the ingredients left suspended in the liquid phase progressively decreases.

Early attempts to overcome these problems varied as to method and effectiveness. A patent issued to Beard in 1954 (17) claimed successful formulation of suspensions of powders of terramycin hydrochloride, viral agents used to inoculate chickens against different virus diseases, and sodium bicarbonate. Beard relied on simple suspension of the dry finely powdered materials in various combinations of halocarbon propellants which possessed "a specific gravity such as to inhibit agglomerative sedimentation of the powdered material." No mention was made of optimum particle size or use of additives to stabilize the suspensions.

Several methods have been reported which make use of additives, such as isopropyl myristate (51, 52) or light mineral oil (217), to provide "slippage" between particles, to lubricate the component parts of the aerosol valve, and to increase adherence of the sprayed particles to skin or other surfaces. Isopropyl myristate was found to be ineffective by Thiel and his coworkers (218) when the suspended powders were dispensed with a metering valve. Bird (23) discovered that he could prepare sprayable antibiotic compositions by converting the crystalline forms of tetracycline hydrochloride and chlortetracycline hydrochloride to their amorphous calcium salts. He further specifies that the salt be dry and finely subdivided so that at least 95% of it is less than 15 μ in diameter.

A satisfactory suspension of high solids content without particle agglomeration has been reported by Geary and West (73). This suspension contained bulking agents such as Santocel 54 to fill the void space between suspended particles.

The various factors which are responsible for particulate stability in aerosol suspensions and the relative importance of these factors have been thoroughly elucidated in a patent issued to Thiel, Porush, and Law, assigners to Riker Laboratories, Inc. (219). Their findings have been confirmed by Kanig and Cohn (107) in a parallel study.

Variables Affecting Particulate Stability.—There is general agreement in the literature that the state of subdivision of the solid is critical not only for proper utilitization of the aerosol but also as a factor in the stability of the suspension. It is obvious that the particle size of the suspensoids must be at least smaller than the smallest valve orifice and small enough to fulfill the operational objective of the product. However, the more finely subdivided the powder is made, the more free surface energy is generated. Molecules inside a powder particle are acted upon by balanced forces produced by molecules surrounding them. On the surface of each particle, however, the molecules are acted on by inward forces only, and this produces an imbalance of forces at the surface. Decrease in particle size with the attendant increase in surface area results in a surface energies to mass of particles ratio so great that when particles collide they adhere strongly. This is probably one of the primary causes of agglomerate formation in pharmaceutical aerosol suspensions where 95% of the particles are generally under 10 μ in diameter and none are larger than 50 μ.

Another variable is the moisture content of the system. The degree of sensitivity of different suspended materials has been shown to vary directly with the solubility of the solids in water. Observations made on the rate of agglomeration of different suspended materials indicated that

the more soluble the material is in water, the more rapidly it agglomerates in the presence of moisture (107). Since water is immiscible with the propellants, any moisture in the system would tend to be attracted to the more polar solids, and the more water-soluble compounds would undergo physical changes leading to agglomeration. In the presence of a sufficient amount of water, growth of crystalline materials could take place. Crystal growth in this instance is due primarily to the conversion of a less stable configuration to one which has a lower energy state. This has been demonstrated in nonpressurized liquid suspensions of finely divided crystalline materials (99). No significant differences in rate of agglomeration were observed when water was introduced into the system, either by being added to the dried powders before they were suspended in the propellants or to the completed suspension (107).

Relative polarity between suspensoid and suspending medium has an influence on particulate agglomeration. The greater the difference in polarity between the powder and the liquid, the more difficult it is for the liquid to wet the surface of the solids and the greater the tendency for moisture to cause agglomeration.

An additional variable is the relative density of suspended powders and propellants. Where possible, equalization of the densities of the suspension medium and the particulate solids tends to reduce the rate of settling. However, results obtained with suspensions in which the density ratios had been adjusted to unity were inconclusive as to the role of equalized densities in preventing particulate agglomeration (107).

A beneficial result of the adjustment of densities was observed in a decrease in the amount of caking which occurred on the inner walls of the container. Thiel, *et al.* (219), found that the addition of a sufficient quantity of an auxiliary finely divided solid of density greater than that of the liquid phase will prevent the surface spread of lighter powders, thus avoiding "caking out" and its associated drawbacks. The auxiliary powder may be of any chemical type, provided it is compatible with the other ingredients and insoluble in the propellant. It may also serve as a desiccant for the system as when anhydrous sodium sulfate or calcium chloride is used. A formula for the calculation of optimum concentration of the auxiliary powder is provided by these authors.

Control of the Variables.—The use of surface-active agents in overcoming the problems of suspension stability has been recognized in several fields. There is ample evidence in the literature to indicate that surfactants can be utilized as protectives and stabilizers in the preparation of suspensions (12, 13, 40, 99, 182, 201).

It was found that surface-active agents could be used as dispersing agents in aerosol systems and serve several purposes. By becoming oriented at the solid-liquid interface, a surfactant provides a physical barrier to agglomeration caused by free surface energy, differences in polarity, and the presence of moisture. The ultimate and desired effect is therefore the maintenance, in the suspension, of particles at approximately the original size. Liquid, nonionic, lipophilic surfactants which are readily soluble in the propellants were observed to be the most effective in a range of from 0.25 to 5% by weight of the total composition. Surfactants such as sorbitan trioleate, sorbitan sesquioleate, polyoxyethylene sorbitol tetraoleate, and polyoxyethylene sorbitol pentaoleate have been found satisfactory in providing stable suspensions, provided that no more than 300 p.p.m. of water are present in the total aerosol system. Maximum water content tolerated without particle agglomeration will vary with the solubility of the suspensoid in water, the surfactant employed, and its concentration in the solution.

Additional work is still required in this area since there are some factors inherent in the use of surface-active agents which offer disadvantages. It is conceivable that some surfactants may partially solubilize a suspensoid in the halocarbon propellants. This might lead to changes in crystal configuration and result in growth or agglomeration. Partial solubility and recrystallization may lead to a gradual deposition in the orifices and expansion chambers of the valve and cause a leaking or clogged valve (16). Maximum solids capacities of such systems are somewhat limited. An increase in total solids content requires correspondingly larger quantities of the surfactant. In suspensions containing 25% solids or higher, the films of sprayed powder are oily or greasy in nature. Depending on the intended use of the suspension, this effect may be a nuisance or an advantage.

Emulsions

More fundamental work has been done in the area of emulsions than in any other aerosol form because emulsions have offered the greatest opportunity for new and useful applications of pressurized systems and because the principles which control conventional emulsions are easily applicable to aerosols. In addition, the pressurized emulsion presents features not usually or easily obtained in ordinary emulsions since there

is greater versatility to be found in characteristics of the emulsion spray.

Emulsions which would otherwise be judged as being poorly formulated are vastly improved by the mechanical shearing action of the valve or the flashing off of dissolved propellants. In fact, the mechanical forces brought into play when an aerosol emulsion is propelled from the container may be considered to be the equivalent of *in situ* homogenization. Moreover, the propellant plays a more vital role in aerosol emulsions than in other aerosol forms. Not only does the propellant supply the energy for the self-propelling features of the aerosol, but it also has a decided effect on the spray character by modifying foam density, foam strength, drying time, and other important features (34, 174, 207, 212). These features have accounted for the phenomenal success of aerosol shaving lather which was invented and developed in 1949. The product as described in the original patent (168) consists of an aqueous soap solution and a liquefied propellant in a container with a valve-controlled opening. The propellant is only slightly soluble in the soap solution but is readily emulsified in it to produce what might ordinarily be evaluated as a poor emulsion. When the emulsion is dispensed, however, it develops a lather possessing desirable characteristics as the propellant rapidly expands and flashes off.

Aerosol emulsions do not produce lathers or foams only. Spray characteristics may be varied from a fine mist spray to a stream or a foam, depending on the type of emulsion formulated, the emulsifying agent, the propellant composition and proportion, the presence and nature of additives, and the type of valve and valve actuator used on the container (91, 174, 207, 224). Aerosol emulsions are classified as oil-in-water or water-in-oil types but take on the extra dimension imposed by the propellant. In o/w emulsions, the propellant becomes dispersed as fine droplets in the internal phase and has a great effect on the characteristics of the foam. Stiff, dry, resilient foams are obtained with a high vapor pressure propellant and a high concentration of propellant. Conversely, soft, wet, flat foams are produced with lower vapor pressures or lower concentration of propellant in the internal phase (216). These effects are demonstrated by use of either the liquefied halocarbon propellants or the hydrocarbon propellants such as propane, butane, and isobutane. Water-in-oil emulsions produce unstable wet foams since the propellant is in the outer phase and flashes away without creating the voluminous foam which it does when it is dispersed in the internal phase.

It is only when a compressed gas, such as nitrogen, is used that the emulsion characteristics remain substantially unchanged. Emulsions are merely expelled from the container by the piston-like action of the inert and undissolved gas. This offers no advantages over the conventionally contained emulsion but imposes many shortcomings which are characteristic of the compressed gases.

Nonaqueous emulsions and foams have been developed for use in aerosols. By replacing water with different polyhydric alcohols or with glycol derivatives, Sanders (208) has produced foams which may be varied widely to suit any particular application. Extremely stable foams may be produced because the glycols do not dry rapidly. In some instances, these foams may approach creams in their consistency, or they may be varied to achieve "quick-breaking," unstable foams. The range of foam characteristics together with the nonaqueous feature of these emulsions makes them suitable for pharmaceuticals where water is not desired for reasons of stability, or where it is contraindicated in the condition being treated.

Emulsifying Agents.—The selection of aerosol emulsifying agents is still being made on a rather haphazard and pragmatic basis. Considering the wealth of scientific information available regarding the selection, use, and evaluation of emulsifying agents for nonpressurized systems, it is surprising to observe how little of this has been applied to aerosol emulsions. While it is true that hundreds of surfactants have been evaluated for this purpose, most of the work has been concerned with effects rather than causes. As a result, only generalized rules-of-thumb are available as guides when a new pressurized emulsion is contemplated. Moreover, even these guide lines are inadequate in predicting the behavior of an emulsifying agent in a system other than that in which it was tested (211).

It has been advocated (106) that methods of research and evaluation which have proved reliable for nonpressurized emulsions should be adapted for use in pressurized systems. Proceeding along these lines, effective relationships have been observed between emulsion stability and HLB values of surfactants (Fig. 5) and kauri-butanol values of propellants (Fig. 6). Preliminary evidence exists to indicate that the relative polarity of propellant-surfactant solutions exerts an influence on pressure emulsification (108).

No reports have appeared to indicate that

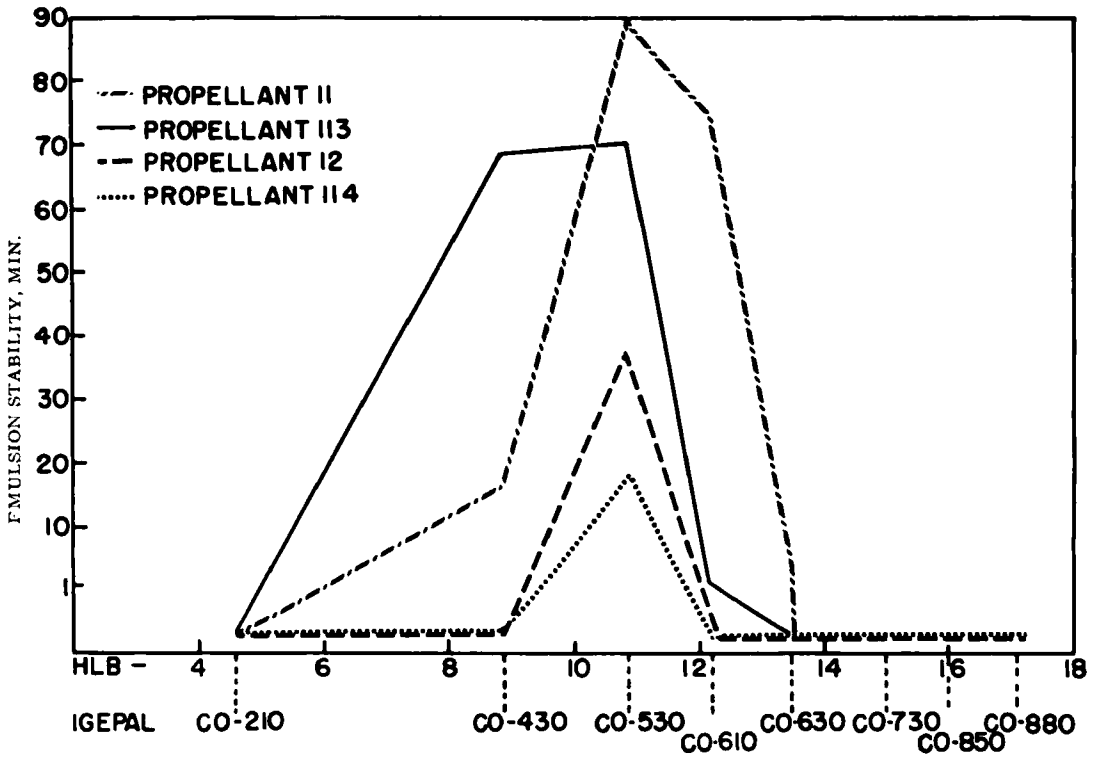

Fig. 5.—Relationship between HLB values of Igepal CO series of surfactants and emulsion stability of propellant-water systems. [From Reference 108.]

studies have been conducted on the use of emulsions in pressure systems as vehicles for insoluble suspended matter. Aerosol emulsion-suspensions offer interesting possibilities for application in topical and related therapeutics.

INHALATION AEROSOLS

Inhalation therapy, it has been said (122, 141, 163, 185), goes back at least as far as written medical history. Fumes of burning herbs or powders, steam inhalations, and nebulized or atomized solutions of a variety of compounds have been in use for the direct therapy of bronchial and pulmonary diseases for many decades (24). Therapeutic aerosols have been produced by various types of equipment (39, 109, 195), and improved devices such as inhalers, atomizers, nebulizers, and insufflators (141) have been a prime factor toward increasing the efficiency and acceptability of inhalation therapy over the years. Long before the introduction of the self-contained pressurized aerosols as pharmaceutical dosage forms, many studies had been conducted on the use of medicaments for local therapy of the respiratory tract or for rapid systemic effects through absorption from the inner surface of the lungs. As newer chemotherapeutic agents of interest in inhalation therapy became available, they were investigated for a variety of pulmonary conditions (1, 94, 111, 194). These substances include antibiotics (66, 162), antitubercular drugs, sympathomimetic amines (75, 109), antispasmodics (160, 161), mucolytics (61, 123, 203, 222, 223), surface-active agents (128–130), certain vitamins (notably Vitamin B_{12}) (137, 138), and corticosteroids (74, 167). This partial accounting of pulmonary therapeutics serves to indicate that the aerosol is not nearly so new an approach as one might assume by reading the recent papers describing the potential of the pressure pack in inhalation therapy. It is only when a comparison is made between the utility, dependability, and reproducibility of the devices employed that the advantages of the new type of unit become apparent.

It has been recognized that the physical state of the particles in therapeutic aerosols exercise a critical effect on the site of deposition and absorption from the respiratory tract (1, 109, 145, 150). Therefore it is claimed that the pressurized aerosol dosage form offers the advantages of accurate delivery of a measured dose of aerosolized drugs, particles of which are within a specified range of effective diameter sizes. Reproducibility of amounts delivered from this dosage form is said to be superior to oral liquid preparations

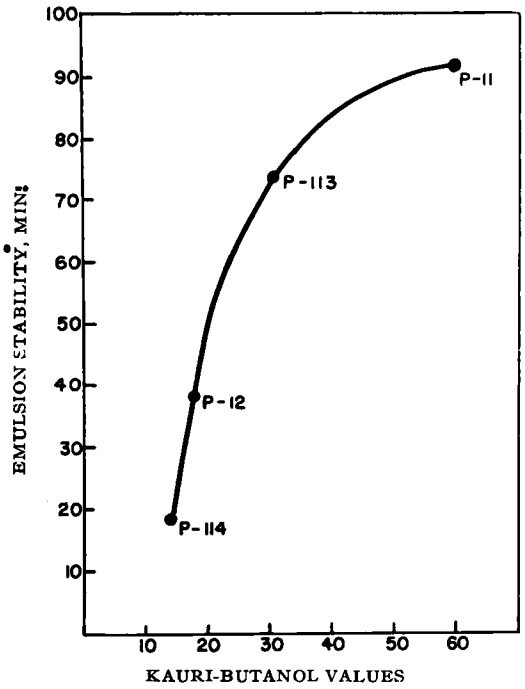

Fig. 6.—Propellant-water emulsion stability related to solvent power of propellants with 0.1% Igepal CO-530. [From Reference 108.]

dispensed by teaspoon or dropper and to compare favorably with weight variations permitted for hard gelatin capsules (159, 241).

Additional unique advantages over other types of vaporizers include: (*a*) a compact container capable of delivering hundreds of single doses, making it the least clumsy of all devices and perhaps the most economical; (*b*) application is rapid and convenient and does not require manipulative skill on the part of the patient; and (*c*) the container is hermetically sealed which makes it tamper-proof and its contents protected against bacterial contamination, moisture, and oxidation or evaporation during periods of disuse. Presumably these factors are sufficient reasons for the reappraisal of the oral inhalation route in therapeutics and for the increased interest in methods for evaluation and control. Enthusiasm for this dosage form has even caused some of its advocates to proclaim that inhaled aerosolized drugs may ultimately replace the parenteral or oral routes of administration in some instances.

This has already been clinically demonstrated in the use of orally inhaled ergotamine tartrate in the treatment of vascular headaches, including migraine and histaminic cephalalgia. The efficiency of this route of administration is said to parallel closely that of injection and to be superior in speed and effectiveness to suppositories, sublingual or oral therapy (64, 79, 90, 202).

To appreciate the potential of this route of administration, it is necessary to examine the critical aspects of the physiology of the lungs on the rentention and absorption of inhaled particles and to relate these to the ability of the aerosol dosage form to meet these requirements. It is conceivable that some of the negative clinical results obtained by oral inhalation methods were due to poor control over the critical variable of particle sizes.

Deposition in the Lungs.—One square meter of lung surface is required for each kilogram of body weight to sustain the life processes depending on the exchange of oxygen and carbon dioxide. In the normal adult this amounts to the area of a tennis court (37). Such an area is encompassed in the small volume of the thorax by subdivision of the inner lung surface into hundreds of millions of alveoli. Within the walls of the alveoli the blood is spread out in a thin sheet, separated from the air by a membrane about 1 μ in thickness. This large area, richly endowed with blood and separated from airborne substances by only a thin membrane, theoretically offers a highly absorptive area for rapid transfer of drugs into the blood stream. Davies has stated "Particles of solid material that deposit on the ciliated lining and the tubes have rapid access to the blood stream. Lung defenses are completely avoided, and immediate toxic action can be exerted, sometimes more effectively than in the case of oral administration. The importance of solubility is demonstrated by the behavior of lead compounds. The dust inhaled by miners of Galena has low solubility and is not dangerous, but the mining and smelting of lead carbonate ore have frequently caused acute lead poisoning by inhalation of the relatively soluble dust" (49).

The chance of an inhaled particle reaching the alveolar regions depends on its size, weight, and shape. The defense of the lungs against retention of any particle is provided by the tortuous nature and decreasing size of the channels through which it must pass, thus making impaction with the walls very probable. After impaction, a particle which is slowly soluble in the mucus on the walls is removed upward by ciliary action. This inertial impaction is an important mechanism in the nasal airways and in the upper lung where the air velocity is fairly high (134). Studies on deposition of particles in curved tubes have indicated that the large particles are likely to be removed from the air stream by this mechanism and that the inertial impaction of the particles is directly proportional to the density and to the square of the particle diameter (116, 125).

Large particles which may survive inertial influence in the upper respiratory tract will settle downward in the lungs and become deposited by sedimentation in the areas where air velocity is reduced. Here, too, the sedimentation rate is proportional to density and to the square of the diameter.

Particles small enough to penetrate into the alveolar spaces where air velocity is very low are made to collide with the walls by Brownian motion which increases in activity as the size becomes smaller.

Particle Size and Retention.—Interest in particle retention in the lungs has been sharpened by studies in chemical warfare, aerosol therapy, and the hazards associated with industrial dusts and radioactive fallout (5, 6). Papers published in this area fail to agree on the relationship between particle size and retention in the lungs. Mathematical models have been proposed for theoretic calculations of the extent of retention of particulate matter of various sizes in the lungs of man (7, 20, 48, 59, 65, 92, 221, 232). The classical theoretic treatment of retention was made by Findeisen (65). He computed the average dimensions of the adult tracheobronchial tree on a functional concept of structure rather than a strictly anatomic description. His calculations were later modified by Landahl (114), who used slightly different dimensions of the tracheobronchial tree and computed retention values for several different flow rates. The schematic representation of the pulmonary tree used by Landahl is shown in Fig. 7. This indicates that particles larger than 20 μ should fail to go beyond the terminal bronchioles; those 6 μ in diameter would be removed before they reach the lower alveolar ducts, and almost all particles 2 μ or larger are removed in the lower alveolar ducts.

Experimental studies in man with heterogeneous aerosols have been reported (5, 15, 31, 42, 43, 47, 48, 55, 115, 116, 120, 121, 127, 139, 180, 236), while others have designed special techniques for generating monodispersed aerosols in which all the particles are nearly the same size. These were used in respiratory retention experiments (8, 117, 118, 231).

Figure 8 indicates the results obtained by Brown (31), using clay dusts having median diameters from 0.24 μ to more than 5 μ. Employing nasal inhalation techniques, Brown

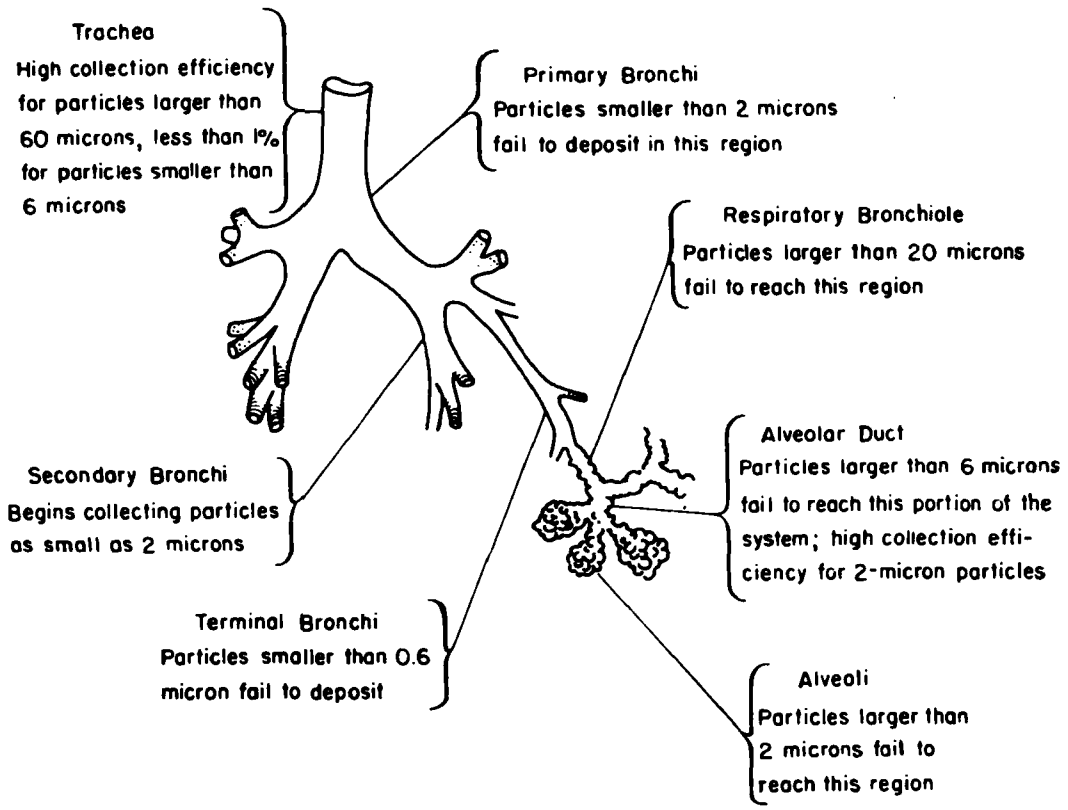

Fig. 7.—Schematic representation of the pulmonary tree showing theoretic retention computed by Landahl. [From Reference 134.]

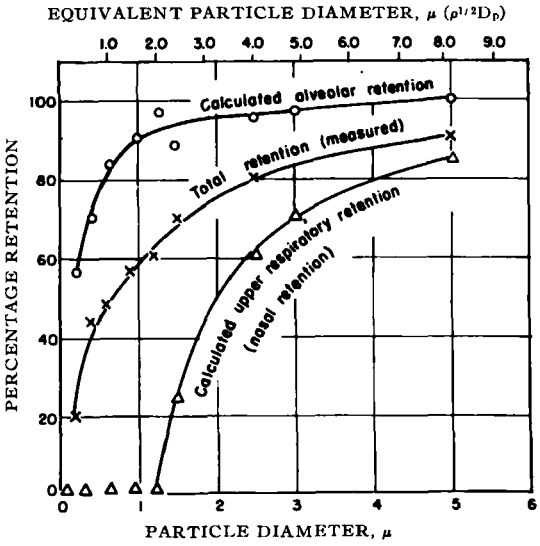

Fig. 8.—Lung retention of clay dusts as a function of particle size. [From Reference 134.]

showed that 85% of the particles of 5 μ in diameter were retained in the upper respiratory tract and that more than 90% of the particles between 1–5 μ in diameter which pass the upper respiratory tract are retained in the alveolar region.

Dautrebande disagrees with such findings and contends "that for ensuring deep penetration into the most efficient part of the lungs of the largest quantity of drugs, the micellary size of the aerosol should be below one micron" (41). He has authored many articles on his studies of methods of generating submicron particles and evaluating their effects and deposition in the lungs (44–46, 50).

The disagreement between these and other workers has not yet been completely resolved. Possible reasons for the conflicting theories and experimental results may be summarized by:

1. Experiments conducted in man or animals may alter normal respiratory mechanics by tracheal catheterization, anesthesia, or use of irritant particles.

2. Differences in the methods of generating aerosolized particles, length of exposure, and methods of detection of expired particles are critical.

3. Either homogeneous or heterogeneous particle size distributions have been used in different studies. Heterogeneous distributions have been criticized because much of the meaning of test results become obscured when an average diameter is used to represent a broad size distribution (134).

4. The possibility exists that materials detected in the lower regions of the pulmonary tree reach there by the indirect route of systemic absorption and circulation.

5. Depending on the efficiency of the aerosol generating system, agglomeration of the particles may occur after leaving the apparatus. Some particles may also increase in size as they encounter the moist air in the lungs while others generated from aqueous or other volatile solutions may decrease in size. If these dynamic changes are not taken into account, erroneous results may ensue.

6. Some investigators have failed to use uniform breathing rates in experiments with humans. In addition to particle size, the next important variable in the retention of particles is the length of time the particles remain in the lungs. The effect of breathing rate is more pronounced for large particles than for smaller ones (134).

7. Methods of measuring particles vary in accuracy and range. As a result, retention values may vary depending on the validity of the method used in measuring the particles.

The entire picture of inhalation therapy is made more complex by the discovery of the existence of a powerful surface-active agent which coats the alveolar spaces in the lungs (37). The substance is thought to be a phospholipid, particularly dipalmitoyl lecithin. It has been found in the lungs of all mammals tested (mouse, rat, guinea pig, rabbit, cat, dog, cow, and man), but not in amphibians (frog and toad), reptiles (snake and crocodile), or bird (pigeon and chicken). It now appears that the surfactant maintains the elasticity of lung tissue and keeps the lungs from collapsing. The surfactant has the further function of assisting osmotic forces acting across the surface of the lungs, and this keeps the film of moisture present there from drawing fluid into the alveolar spaces.

The presence of this powerful surface-active agent in lung tissue fluids may have a profound effect on particulate retention and absorption. This has not been considered in any of the cited studies.

The fact that a surface-active agent is critical to normal lung function must also be taken into account when oral inhalation dosage forms are designed. Substances which are included in the inhalations as active therapeutic agents or for product stability might have a neutralizing or adverse effect on the physiological surfactant. The ultimate results of continuous or prolonged oral inhalation of a variety of substances are open to investigation.

PARTICLE SIZE MEASUREMENT

Particle technology has become increasingly

important in so many diverse areas (204) that the development of particulate fractionation and measurement techniques have been accelerated by the demands of research into fine particle properties and behavior. Methods have been developed for a variety of industrial, meteorological, and biological purposes. Without such methods the study of the role of particulate matter in production, agriculture, and health and disease would be seriously hampered. For the most part, however, many of the particle size analysis methods are of limited value in measuring particles which originate from pressurized systems or which fall into the particle size ranges of interest in the therapeutic applications of aerosols.

Particle size measurement is rarely an absolute procedure. Inherent in almost every method are limitations which are imposed by the physical

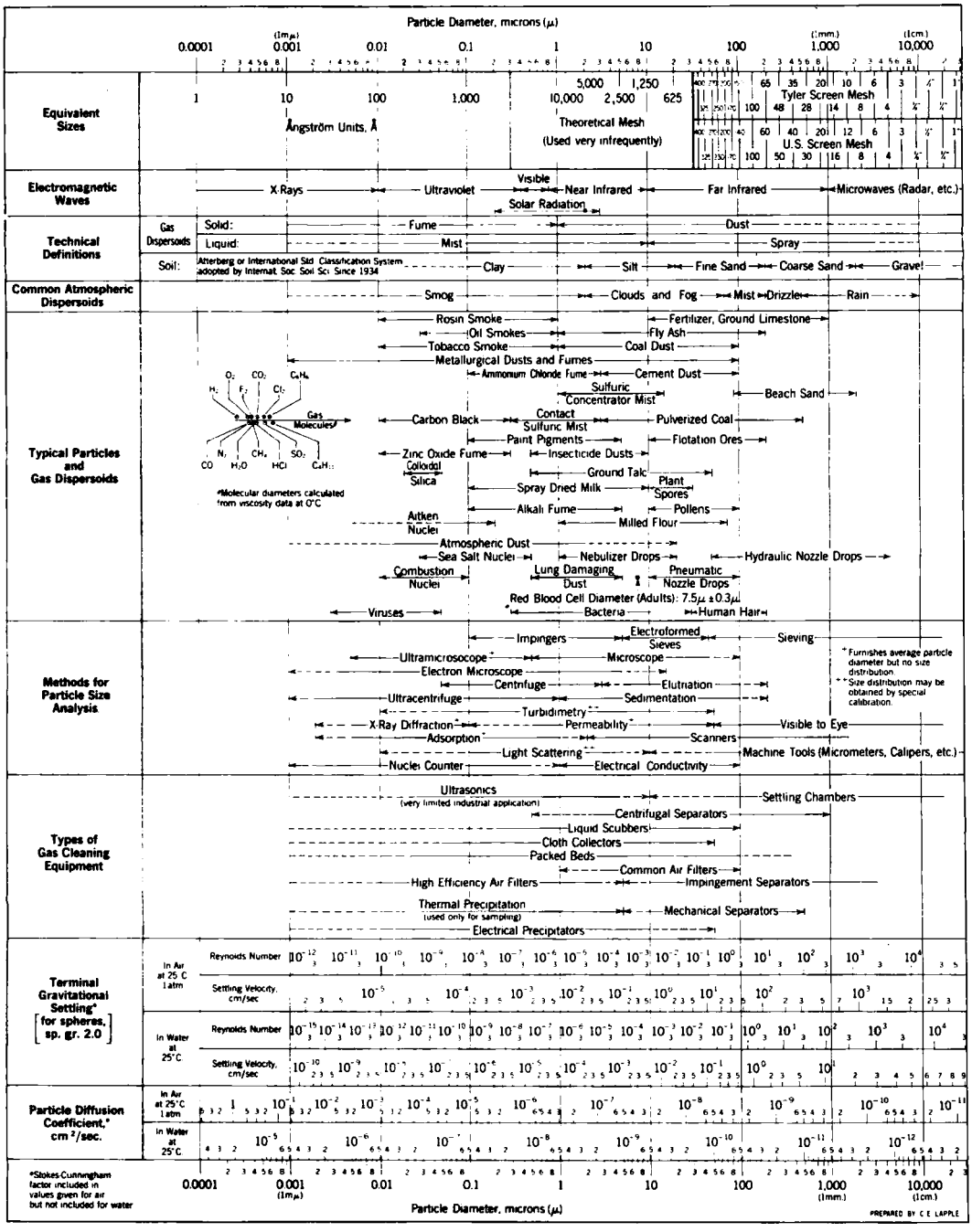

Fig. 9.—Characteristics of particles and particle dispersoids. [From Reference 204.]

principles which are employed. These limitations affect not only the accuracy of the method but also dictate the types of particles which may be measured and their sources. Many of them furnish only average particle diameters but no size distribution, while the size distribution obtained from others may be accomplished only by means of special calibration. The scope of characteristics of particles and an indication of the complexities which exist in the area of particle technology may be ascertained from Fig. 9.

It must also be remembered that almost every technique which has been employed in particle size measurement presupposes an accurate conveyance of a valid and representative sample to the instrument. Particles sampled from a pressurized container must be prevented from agglomerating before they are measured; this aspect of the procedure imposes serious limitations on the types of techniques which may be employed. When little attention is placed on the sampling of particles and the effect it can have on the accuracy of the method, the results obtained are only approximate answers (81).

As a consequence of these limitations, relatively few methods may be considered as being capable of accurately measuring the size range of particles within the framework of aerosol dosage forms and under the restrictions of the unique variables imposed by the generating mechanisms.

The nature of the particle delimits the size analysis method as well. Suspensions of particulate solids in aerosol propellants produce particles essentially unchanged if agglomeration can be avoided as the propellants boil away. Solution-type aerosols offer additional complications in that the droplets are constantly changing size after being sprayed. A reduction in size with time results when the propellants or co-solvents evaporate within seconds and precipitate or crystallize the dissolved solids. Particle size growth may occur when the particles suspended in air are hygroscopic and begin to absorb moisture (77), or when they are liquids under ambient conditions and coalesce upon impact.

Any measurement of aerosol particles must therefore take into account the changes which occur in these dynamic systems after spraying and the possible effects these may have on the accuracy of sampling and size analysis.

Methods of Particle Size Measurement

Microscopic Measurement.—Microscopic techniques have been classified as being among the most accurate of the *direct* methods (*i.e.*, methods in which the actual dimensions of particles are directly measured as contrasted with *indirect* methods which are based on measuring some other property of the particles which is related to their size) (67, 98).

The unique feature of microscopic measurement is that particles are sized directly and individually rather than being grouped statistically by some process of classification. Disadvantages to the method lie in the tediousness associated with the necessity of measuring a sufficiently large number of particles to provide adequate representation.

Limitations in the use of the optical microscope are due to the fundamental laws fixing maximum resolution which set the lower limits of particle size measurement using visible light. This limit of resolution has been reduced by use of ultraviolet light or monochromatic sodium light. Electron microscopy has further reduced the lower limit of resolution to 0.001 μ. However, the upper limit of size is not greater than 10 μ and problems associated with sample preparation reduce the utility of the electron microscope. Ultra-microscopy has been employed to count and measure particles suspended in a gaseous medium but has been supplanted, for the most part, by light-scattering techniques.

Sedimentation Techniques.—Speed of fall under controlled or predictable conditions has become one of the more widely used techniques among the indirect methods of measurement. Application of Stoke's law to free falling particles in liquid or air media is the basis for several instrumental methods of measuring. The physical methods involved in the actual determination of particle size generally conform to the same fundamental principle. If the particles are initially distributed uniformly in a column of fluid at rest, there will be, after a lapse of time, a density gradient in concentration of particles along the height of the column. Determination of this density gradient enables the proportion by weight of the various sizes of particles to be calculated. Any property of the suspension which is a function of the density or particle concentration may thus be used to determine the size distribution. A discussion of the methods of determining variation in particle concentration has been published by Work and his associates (184, 235). Additional theoretical concepts advanced by Heywood (97) and Egolf and McCabe (58) are useful in this area.

A sedimentation balance, described by Oden (142), provides a method of directly weighing particles at selected time intervals as they fall in a liquid system. For continuous observations, an automatic recording-type balance is available.

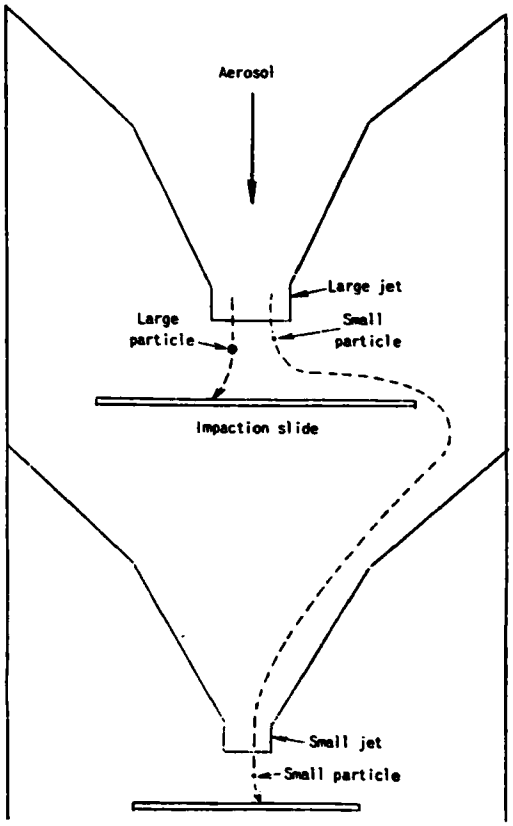

Fig. 10.—Schematic diagram showing principle of the cascade impactor. [From Reference 154.]

Additional refinements of the sedimentation method have been reported. The pipet method, advocated by Schweyer (183), involves the withdrawal of samples of the dispersion at predetermined depths. Evaporation of the solvent then yields concentration data from which particle size may be derived.

A hydrometer bulb method, advanced by Bauer (14), measures changes in density induced by variations in concentration. This is an advantage over the pipet method in that it does not permanently withdraw a portion of the suspension under study. A disadvantage of this method is that the introduction of the hydrometer bulb tends to disturb the settling particles. The Diver method (102) overcomes this disadvantage by utilizing small vessels of varying known density which assume equilibrium positions in the suspension system according to the density gradient that forms as the particles settle.

An innovation in the area of gravitational sedimentation is the Carey and Stairmand photosedimentometer (33) which photographs the tracks of particles as they fall in a dispersion system. The size determination is derived from the length of the photographic track, which is an indication of the distance traveled by the particles, and the time of exposure of the photograph.

The lower limit of size to which gravitational sedimentation methods can be applied is controlled by temperature variations causing circulatory currents in the suspension, by flocculation of particles during the progress of sedimentation, and by diffusion or Brownian movement of the very small particles. All these factors, plus the extreme length of time required by these methods, make it advisable to shorten the sedimentation process as much as possible. This may be accomplished by substituting centrifugal force for gravitational acceleration. The principles of sedimentation are unaffected, except that the factor g in Stoke's equation becomes a variable according to the speed and radius of rotation of the particle within the centrifuge. For example, using gravitational sedimentation, it would require 1057 minutes for a 1-μ diam. particle to fall 5 cm. but would only require 3.3 minutes to traverse the same distance when using a centrifugal speed of 2000 r.p.m. Instruments embodying the centrifugal principle have been described by Schachman (181) and Kamack (105).

Another process which considers the velocity of motion relative to a fluid for the determination of particle size is that of elutriation. Elutriation differs from sedimentation techniques in that the particles are held stationary in space through the attainment of balance between the gravitational

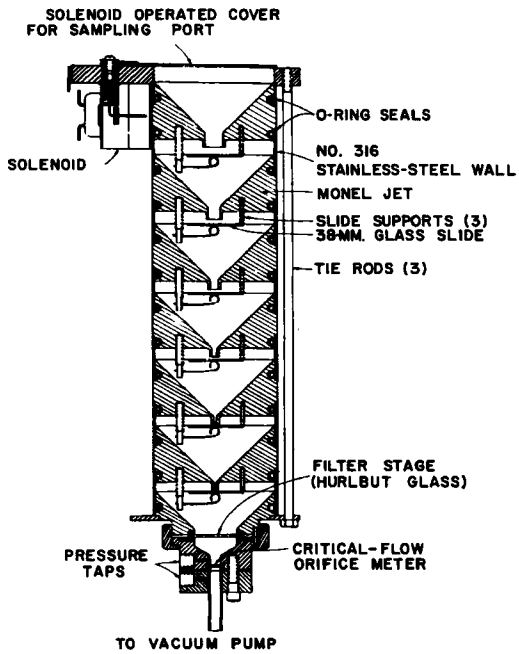

Fig. 11.—Multi-stage cascade impactor. [From Reference 154.]

forces acting on the particles and the viscous forces between the particles and an upward moving column of fluid or gas (78, 170).

Impaction and Inertial Techniques.—Methods in this category for the sampling and estimation of particles in the sub-sieve range are based on laws governing the trajectories of particles in fluid streams. These inertial principles, which are a function of size, have permitted the development of a wide variety of instruments. Included are techniques embodying cyclone deposition, impactor sampling, inertia separation, and electrostatic and thermal precipitation. In a number of these methods it is possible only to effect a separation of a sample into a series of size fractions. The exact particle size of each fraction must subsequently be determined by additional means. In other instances, a size analysis can be obtained directly by theoretical treatment or prior calibration of the instrument.

Impaction devices utilize the deposition characteristics of fine particles in a moving air stream when directed past obstacles of defined geometric form, or in being forced from a jet device onto a plane surface. The cascade impactor has received considerable attention for use with aerosol particles. This device, described by Pilcher and his co-workers (154), forces particle-laden air at very high speed and fixed rate through a series of jets (each smaller than the preceding one) onto glass slides; impaction takes place in a series of stages. The velocity of the air stream and particles suspended in it are increased as they advance through the impactor. As a result, the particles are classified by impaction on the different slides, with the larger particles on the top slides and the smaller particles on the downstream slides. Figure 10 illustrates the basic principles in operation. Modifications in the cascade impactor method have been made to increase the sharpness of classification and to extend the range of particle sizes (135) (Fig. 11). Additional impaction devices employing similar principles have been described by Owens (148), Gurney (84), and Sawyer and Walton (179). Extensive theoretical treatment has been reported by Ranz and Wong (165), Gillespie and Johnstone (76, 103), and Wilcox (229, 230). A small light compact portable cascade impactor for industrial use has been designed and studied by Brink (28). An instrument which operates on similar inertial principles to those of the cascade impactor has been described by Tillotson (220). This variation may be adapted for automatic readout of size distribution by means of light scattering techniques and electronic counters. The method is claimed to be able to produce a complete particle size distribution in a few minutes.

The most serious deficiency of the cascade impactor is the relatively large sample required for analysis. Porush, *et al.* (159), have indicated that this makes determinations of particles emanating from single doses of most aerosols impossible by ordinary analytical methods.

Thermal Precipitation.—The movement of fine particles in a thermal gradient has been described by Watson (225). The underlying principle depends on the introduction of the particles into a region between two bodies of differing temperatures. The particles will move toward the colder body and be deposited on it since the particles are affected by a force directly proportional to the temperature gradient and inversely proportional to the absolute temperature (197). Watson's instrument (225) was capable of measuring particles of 5 μ or smaller, but size distribution was possible only by resorting to microscopic methods of measurement.

Electrostatic Precipitation.—Determination of particle size by the estimation of the magnitude of charges on individual particles has been studied. The charges are either naturally present or are induced on the particles. The magnitude of the charge is directly related to the size of the particle (56, 112, 119). This method has been criticized by Orr and Dalla Valle (147) as being capable of producing erroneous size distribution due to the chance of precipitating more than one size particle at a given point. Instruments which have been developed in attempts to overcome this shortcoming are described by Yoshikawa (240) and Guyton (85). A more advanced and accurate method described by Berg (19) suspends the particles in a conductive liquid which flows through an aperture with a simultaneous flow of electrical pulses (each pulse being proportional in size to the volume of the particle originating it). The pulses are amplified, scaled, and counted to provide data for plotting cumulative particle frequency against particle size. The size range claimed for the instrument is 0.5 to 200 μ diameter at a counting rate of 6000 particles per second.

An excellent review of the theoretical consideration involved in the determination of aerosol particle size distribution by precipitation of charged particles is offered by Drozin and La Mer (56). These authors also describe a method utilizing this principle which takes about 20 minutes, including the necessary calculations, and is carried out with relatively inexpensive equip-

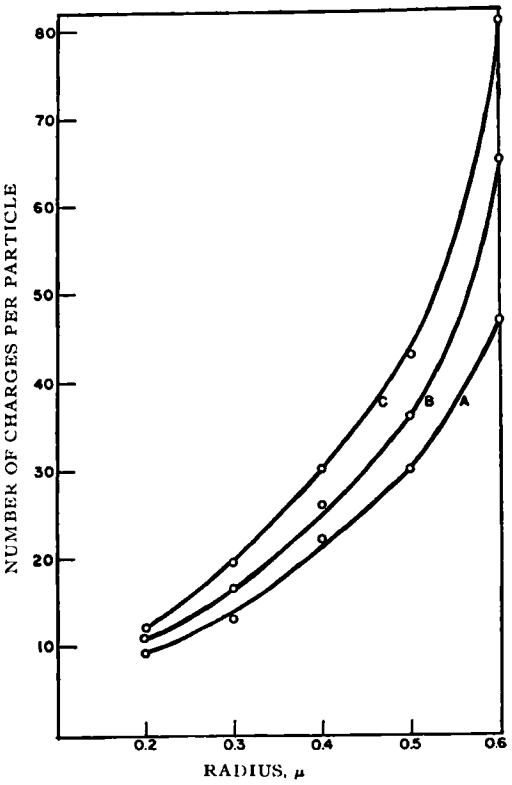

Fig. 12.—Number of charges per particle *vs.* particle radius. Stearic acid aerosol particles charged with current of 40 μa. (*A*); 80μa. (*B*); and 120μa. (*C*). [From Reference 56.]

ment on particles in the submicron range above 0.2-μ radius. Figure 12 illustrates some of the results of their study.

Optical Methods.—The characteristic optical behavior of small suspended particles forms the basis of several indirect methods for assessing particle size. Observations of suspended particles transversed by a beam of light disclose the ability of the particles to scatter, absorb, or reflect the incident radiation to a specific degree. These phenomena have been investigated, and it has been found that the degree is dependent upon the size of the particles, among other variables.

Theoretical treatment of these properties has led to the formulation of equations based upon light scattering and light transmission which permit the evaluation of particle size and cloud concentration (146, 198).

Light transmission or extinction methods are based on the Beer-Lambert law (25) and an instrument utilizing this principle is described by Sinclair (198). The instrument measures the extent to which a beam of light of known intensity is diminished as it emerges from a suspension of particles in gaseous media.

The principles of light scattering formulated by Mie and Rayleigh have been used more extensively in fine particle measurement by Sinclair and La Mer (200). Rayleigh's equations state that the intensity of light scattered from a particle varies as the sixth power of the particle radius for particles which are very much smaller than the wavelength of light, approximately 0.5 μ. The upper limit of size applicable to these equations is about 0.03 μ radius, therefore, these equations have a restricted value for the study of general particulate systems. In the case of larger particles equal to or greater than the wavelength of light, the equations of Mie apply. Here the angular distribution of scattering intensity varies as a function of the scattering angle, and the complexity of the mathematical calculation of size increases markedly with increasing particle size. Consequently, since the computations are massive and difficult and the equations for these larger particles are applicable to spherical monodisperse systems only, these theoretical methods do not lend themselves easily to universal application for particle size measurement. The value of light scattering, however, is apparent as a method of detection of changes in cloud concentration. For this application, light scattering techniques are more than sufficiently sensitive and have been found to be of great value (82, 83, 199).

Dimmick, *et al.* (53), have described an instrument employing light scattering techniques for measurement of small particles. The method is rapid, accurate, and reproducible and depends on an analysis of the change in intensity of a light beam projected through an aerosol cloud under turbulent conditions.

The settling rate of a monodisperse aerosol in a closed chamber under turbulent conditions (stirred sedimentation) is an exponential function of concentration defined as

$$\frac{N}{N_0} = e^{-Vt/H} \qquad \text{(Eq. 3)}$$

where N = concentration at time t in minutes, N_0 = concentration at time zero, V = Stoke's velocity of fall in cm./min. for a given particle and density expressed in Gm./cm.^{3}, and H = the effective height of the chamber.

Being exponential in nature, the log of the concentration plotted against time results in a straight line. The same slope is obtained whether the concentration is expressed in terms of number, geometric area, or volume (related to mass).

The slope is then defined in terms of the half-life ($L\ ^1/_2$) and the authors provide a nomograph which relates particle size and density to half-life for a chamber height of 100 cm. This instrument was employed in measuring particle size of iso-

proterenol and epinephrine aerosol suspensions and the distribution of particles was reported by giving the mass median diameter (159).

A similar approach has been developed by Mintzer (133) and has been used in a study on stability of aerosol suspensions (107). The instrument consisted of a separate sedimentation chamber in which the aerosol cloud was mechanically stirred. Air-tight connections continuously carried particles from the sedimentation chamber to the cell of an extremely sensitive light-scattering photometer. The cell was modified so that the particles could pass out of the instrument after being measured. Readout of phototube signals was accomplished by means of a wide-strip recorder (Fig. 13).

A portion of the recorder chart for a typical determination (Fig. 14) indicates the response to samples of particles withdrawn from the sedimentation chamber. Figure 15 is a plot of the slopes from which particle diameters were calculated using Dimmick's equations.

O'Konski and Doyle (144) have also measured aerosol particles by using light scattering coupled with an electronic particle counter in a single instrument. Fisher, *et al.* (67, 68), have described an instrument which they call an "aerosoloscope." The procedure involves the massive dilution of the airborne particles so that flow rate is controlled and each particle passes through an illuminated region where it produces a pulse of scattered light. The light scattered by each particle is received on a photomultiplier which gives rise to an electrical pulse, the amplitude of which is related to the size of the particle. The counts are sorted and registered separately on a bank of 12 electromechanical counters. Aerosol particles ranging from 1–64 μ in diameter can be measured at a maximum rate of 2000 per minute.

Patents have been issued to O'Konski (143) and Mumma (140) in which electrical impulses are used to measure particles illuminated by an incident light source.

Another aerosol particle analyzer employing right-angle light scattering was recently reported (242). This instrument is said not only to measure particle size distribution but also count them.

Size Analysis of Droplets.—Size analysis of water aerosols or droplets of completely volatile components is difficult because the droplets evaporate quickly in room air. "For this reason," state Reif and Mitchell (169), "most methods of size analysis are indirect, in the sense that water droplets are allowed to hit a suitably prepared surface where they leave a permanent imprint. However, evaporation may occur before the droplets reach the sam-

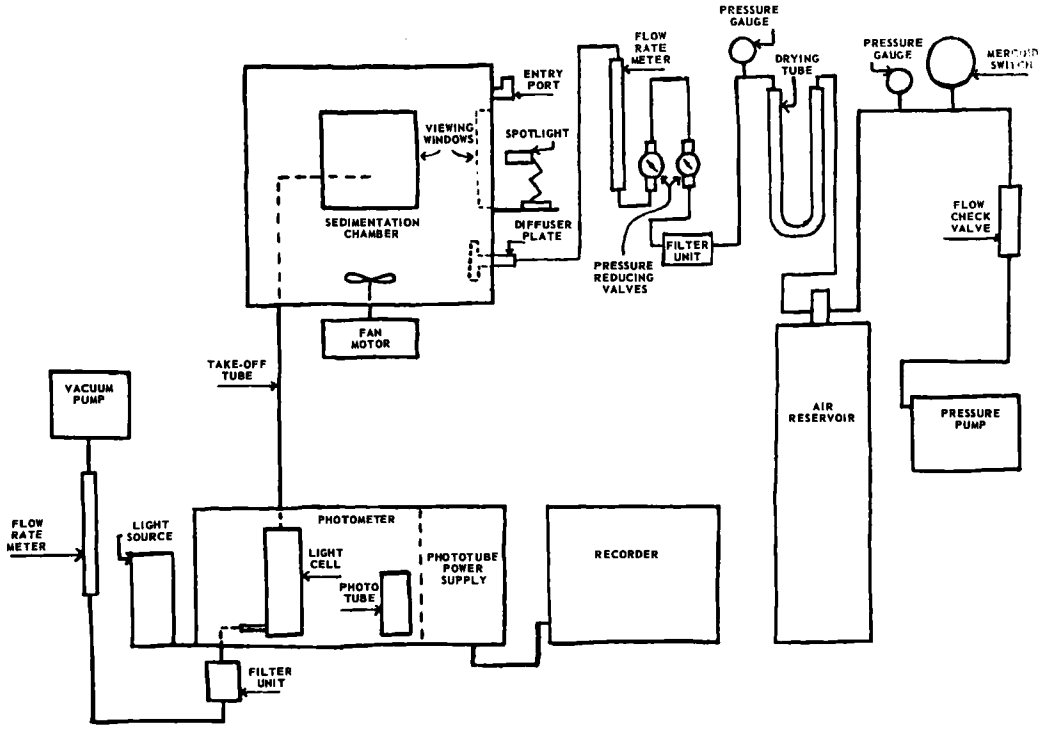

Fig. 13.—Schematic diagram of apparatus employing stirred sedimentation and light-scattering photometer for measurement of particle size. [From Reference 133.]

pling surface and only the largest droplets may carry sufficient momentum to penetrate it. Moreover, a round droplet is smaller than the flat imprint that it leaves on the sampling surface. Therefore, a calibration factor must be determined to convert the measured diameter of the flat imprint into the diameter of the round droplet that originally made the imprint." In recognition of this problem, these authors report an original study involving a vertical sedimentation chamber and the measurement of the effluent aerosol by means of a spectrophotometer. Unfortunately, their method is found to be applicable only to nonvolatile aerosol liquids. However, their report offers an excellent review and laboratory comparisons between six different methods which have been advocated for aerosol droplets. These methods are: (*a*) photographic film coated with polyvinyl alcohol which retains imprints of water droplets that hit it (62, 63); (*b*) a microscope slide covered with an even deposit of amorphous methylene blue powder which is dissolved by water droplets at point of impact and push it to the periphery of a circle (125); (*c*) a film of magnesium oxide which will leave pits proportional to the size of the droplets that hit it (125); (*d*) water droplets are "fixed" by allowing them to impinge on a viscous liquid such as oils or jellies (153, 199, 237); and (*e*) a lens method whereby aerosol droplets are permitted to impact on a scrupulously clean slide and form plano-convex "lens." The diameter of the equivalent spherical droplet can be calculated from three

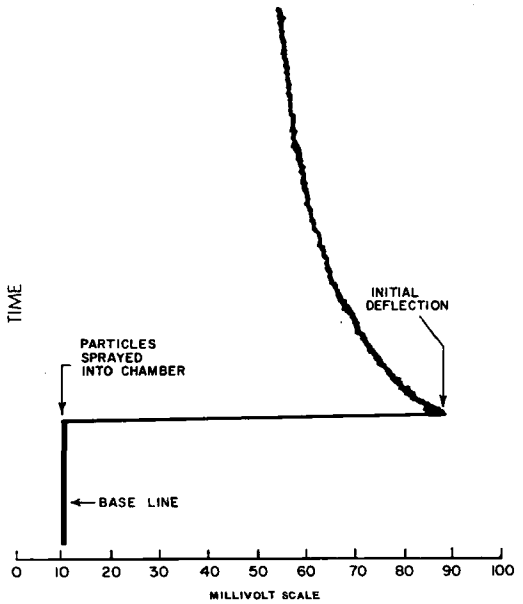

Fig. 14.—Section of strip recorder chart indicating phototube responses to initial stages of particle cloud settling. [From Reference 133.]

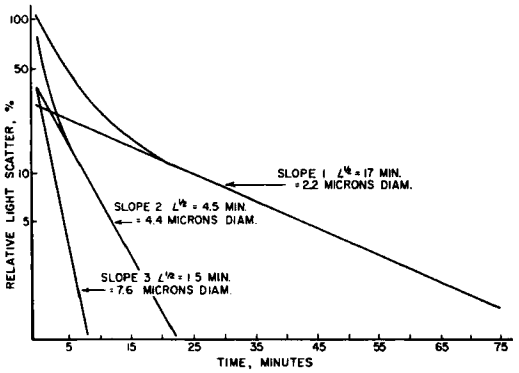

Fig. 15.—Light scattering curves for the determination of mean particle diameter. [From Reference 133.]

parameters. the diameter of the liquid lens, the focal length of the liquid lens, and the refractive index of the liquid (125). Owing to rapid evaporation, this latter method was also found to be suitable for nonvolatile liquids only. The authors conclude that the photographic film-polyvinyl alcohol method is satisfactory and presents factors required to convert the measured imprint to the original droplet diameter.

Standardized Particles.—Since many of the indirect methods of measurement require standardization of the instrument employed, the ready availability of a variety of fine particles of different chemical and physical properties is often welcomed. The Stanford Research Institute at Menlo Park, California, maintains a Particle Bank for both deposits of quantities of surplus particles and withdrawal of samples by research organizations.

EVALUATIVE PROCEDURES

Spray Patterns.—The thermodynamics of spray formation has been discussed by York (239), who analyzed the production of aerosol particulate matter and the energy relationships which exist under the influence of variables encountered in pressurized containers. In order to evaluate the effect of the variables on spray patterns, specialized techniques were developed. These techniques and the apparatus involved have been reviewed by Yeomans and Fulton (238), Dixon (54), Herzka (95), and Root (172). In general, the techniques utilize methods for visualizing and recording spray patterns under standardized conditions. Duplication of results is often poor and, if quantitative values are required, improved methods must be instituted.

Rheological Characterization.—Flow rates and rheological behavior of pressurized materials are important factors in the choice of valve, propellant, and specific formulation components. Consequently, a study of rheo-

logical behavior is of unusual importance when formulations possessing relatively high viscosities are pressurized. The rheological study of an aerosol product is a difficult problem because of different shear rates which are encountered in the various parts of the system. Cohen and his associates have divided the contents of a pressurized product into three rheological zones; within the container, in the narrow orifices within the valve, and at the point of delivery. This comprehensive report (38) analyzes the different shear rates within these zones and describes the use of an extrusion rheometer in measuring the types of flow of several pressure-packed formulations of high viscosities. The relationships between pressure and viscosity have been studied in the dispensing of liquid aerosols of high viscosities and their effect on delivery rates reported by Mina (132). This study also offers a suggested procedure for estimating delivery rates under various pressures.

Analysis of Volatile Constituents.—Gas chromatographic techniques have been shown to effectively circumvent the manifold problems associated with volatile constituents. Root and Maury (175–177) describe their methods of adapting gas chromatography for the analysis of a variety of volatile components under pressure.

Propellant Composition.—Since the vapor pressure exerted by the blend of propellants is critical to the performance of an aerosol, it becomes equally critical to determine accurately the exact composition of the blend. A simple and inexpensive pressure pycnometer has been developed which quickly relates halocarbon propellant composition to density of the blend (214). Tables of vapor pressures and densities as functions of composition at given temperatures are available (213). A similar approach has been reported for the determination of hydrocarbon gases in blends of these gases with liquefied halocarbon propellants (192)

Other Evaluative Procedures.—The Aerosol Division of Chemical Specialties Manufacturers Association publishes a loose-leaf Aerosol Guide which contains descriptions of procedures for a large number of tests developed by the Scientific Committee of the division. Many of these are still classified as "tentative methods" but this compilation is valuable as a reference source to the most reliable methods currently available. The Aerosol Guide also contains a premarketing product check list and information on federal and local acts pertaining to pressure packs.

STERILIZATION OF AEROSOLS

The temperature-pressure gradients of most propellants and the nature of the contents often preclude the use of terminal heat sterilization of the sealed units. The use of ethylene oxide together with liquefied halocarbon propellants has been shown to sterilize effectively the contents of aerosol systems (86, 87). One method (206) consists of presterilizing the containers in hot air ovens, the product and valves with ethylene oxide and ultraviolet light, and the propellant mixture through Seitz bacterial filters. Filling and assembling the antibiotic aerosol is then conducted under aseptic conditions in specially designed filling lines. Sterilization of aerosol products apparently does not pose any serious problems.

SUMMARY

While the development of pharmaceutical aerosols has not been as rapid or widespread as predicted, there is evidence in support of the contention that the aerosol may be counted among useful innovations in dosage forms.

Advances which have been made in evaluating the physical and therapeutic performances of drugs dispensed from pressurized units have made it possible to compare favorably the aerosolized drugs with other methods of administration. The possibility that orally inhaled medicinals may one day become a routine dosage route has been explored but it is clear that a large number of unknown factors must be investigated to insure predictable and controllable results. Other applications of aerosols in treatment of disease must meet the challenge of being compared with currently available medicinals. Unless they offer advantages not now possible with existing dosage forms, pressurized drugs will not be sustained on the basis of consumer convenience alone.

TABLE III.—INTERFACIAL TENSIONS *vs.* HLB VALUES[a]

Surfactant	HLB Values	Interfacial Tension, Dynes/cm. @ 25°C. Exptl.	Calcd.	Deviation (dynes/cm.)
Igepal CO-210	4.6	37.2	34.2	3.0
Igepal CO-430	8.9	18.0	20.0	2.0
Igepal CO-530	10.9	12.3	13.3	1.0
Igepal CO-610	12.2	5.5	8.9	3.4
Igepal CO-710	13.5	4.0	4.7	0.7
Igepal CO-730	15.0	1.5	0.3	1.2

[a] Igepal CO surfactants, 0.1%, in Propellant 11-Water.

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Colorimetric Assay of Nystatin

By J. C. CHANG,† A. B. HONIG, A. T. WARREN, and S. LEVINE

A colorimetric method to assay nystatin both as powder and in pharmaceutical preparations is described. Nystatin is hydrolyzed with sodium hydroxide; the yellow color produced is extracted from a buffered solution into chloroform and measured colorimetrically. Comparison with microbiological assay is shown in a degradation experiment.

NYSTATIN is usually assayed by microbiological methods (1–4). A spectrophotometric method has been reported (5), but it has been known in our laboratories to be subject to ultraviolet absorbing interferences and is not suitable for stability studies.

A yellow color is produced by heating nystatin with sodium hydroxide and this may lead to a colorimetric assay method (6). Direct measurement of the color was tried in this laboratory, but the results did not agree too well with the microbiological assays. Unterman (7) reported a similar method recently.

It was thought that extraneous color was present in the solution after hydrolysis, and color produced by nystatin alone could be extracted into another solvent. Chloroform was found to be the best solvent. It was demonstrated that by measuring the color extracted into chloroform, the results were reproducible and agreed well with microbiological assays, and that degradation of nystatin could be followed by this method.

Preliminary experiments showed that the color produced by the basic hydrolysis of nystatin was unstable in both basic and acidic solution. However, the color was stable for at least 16 hours if the pH was kept at about 6.5. In order to maintain this pH, a citrate buffer was used and found to be satisfactory. Acid neutralization using bromthymol blue as indicator also was tried, but this was abandoned because the indicator was extracted into chloroform.

The time of color development was studied. The intensity of color reached a maximum in 2 minutes at boiling-water temperature and decreased rapidly upon longer heating (see Fig. 1); therefore it was necessary to quench the reaction in an ice bath after maximum color was obtained.

One 10-ml. portion of chloroform extracted about 95% of the color produced by nystatin, and the color was virtually completely extracted with two 10-ml. chloroform extractions. The chloroform extract was turbid due to the presence of water, but the turbidity was removed by adding alcohol. Drying agents were tried but were not as satisfactory.

Different solvents for nystatin were tried, among them methanol and isopropyl alcohol. Although hydrolysis occurred in all the solvents tried, dimethylformamide was chosen because nystatin dissolved in it readily.

EXPERIMENTAL

Reagents

Ethanol, U.S.P. (or SD3A, if colorless); dimethylformamide, reagent grade; chloroform, reagent grade; 1 *N* sodium hydroxide.

Citrate buffer: prepared by dissolving 210.15 Gm. citric acid and 29.4 Gm. sodium citrate, both reagent grade, in water to make 1 L. of solution

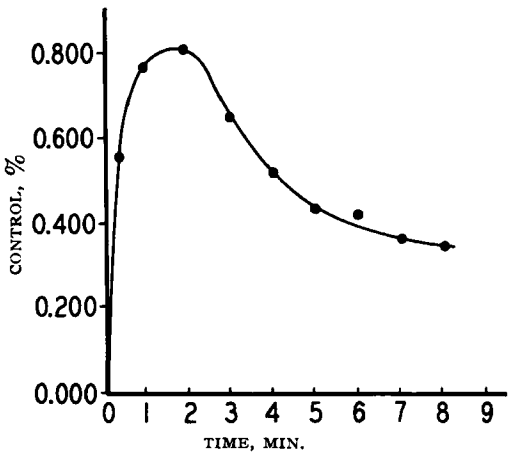

Fig. 1.—Color development of basic hydrolysis of nystatin (5080 units in 5 ml. aliquot).

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The pH of this solution should be 2.3 ± 0.2. If not, 10 *N* sodium hydroxide or 60% citric acid was added to adjust the pH.

Dilute buffer: prepared by diluting 20 ml. of the citrate buffer to 100 ml. with water.

Procedure

A solution of nystatin in dimethylformamide, representing about 700 u./ml., was prepared as directed in *Preparation of Samples*. To a test tube was added 3 ml. 1 *N* sodium hydroxide. Then a 5-ml. aliquot of nystatin solution was added along the side of the test tube in such manner that the nystatin solution formed a layer floating on top of the sodium hydroxide solution. The solutions were quickly mixed by a slight shaking motion, and immediately the test tube was placed in a boiling water bath for exactly 2 minutes. Then the test tube was cooled in an ice bath for 2 minutes, and the contents immediately transferred to a 60-ml. separator containing 6 ml. of dilute buffer. The test tube was rinsed with water, combining the rinsings in the separator. The yellow color was extracted twice with 10 ml. of chloroform, shaking 1/2 minute each time, and the chloroform extracts were withdrawn into a 50-ml. volumetric flask. Ten milliliters of ethanol was added to the flask, and chloroform was added to the mark. After mixing, the absorbance at 385 mμ was measured on a Beckman-DU spectrophotometer in 1-cm. cells, using chloroform to adjust to zero absorbance.

Duplicate aliquots of each sample solution were assayed simultaneously with duplicate aliquots of a standard, and the potency of the sample was calculated from that of the standard by a simple proportion. Although the procedure was standardized, the samples were always assayed with a standard in order to obtain best results.

Preparation of Samples

Powder.—A weighed amount of powder, representing about 70,000 u. was shaken with 50 ml. of dimethylformamide in a 100-ml. volumetric flask for 20 minutes, and dimethylformamide was added to volume.

Cream.—An amount containing about 70,000 u. of nystatin was weighed into a 50-ml. centrifuge tube and was shaken twice with 10 ml. of dimethylformamide for 1 minute. After each shaking the tube was centrifuged for 15 minutes, and the super-

TABLE I.—ASSAY OF NYSTATIN AS THE POWDER AND IN PHARMACEUTICAL PREPARATIONS

Sample	Colorimetric Assay[a]	Average	Microbiological Assay[b]	Spectrophotometric Assay[c]
Powder 1, u./mg.	3,510			
	3,550	3,530	3,580	3,630
Powder 2, u./mg.	3,890			
	3,840			
	3,860			
	3,840	3,860	3,830	3,870
Powder 3, u./mg.	3,750			
	3,770			
	3,720	3,750	3,330	3,780
Powder 4, u./mg.	3,610			
	3,720	3,670	3,500	3,860
Powder 5, u./mg.	3,350			
	3,260	3,310	3,220	...
Powder 6, u./mg.	3,810			
	3,750	3,780	3,690	...
Powder 7, u./mg.	3,760			
	3,740	3,750	3,550	...
Cream 1, u./Gm.	128,200			
	127,700	128,000	117,000	...
Cream 2, u./Gm.	123,600		128,000	...
Ointment 1, u./Gm.	101,600			
	100,600	101,100	108,000	...
Ointment 2, u./Gm.	110,300			
	113,100			
	111,300	111,600	116,000	...
Capsule 1, u./cap.	284,100		306,000	...

[a] Each value represents an individual assay with an independent sample preparation. [b] Assayed by the agar-diffusion method using *Saccharomyces cerevisiae*. [c] Assayed by measurements of absorbance at 291 mμ (8).

TABLE II.—DEGRADATION OF NYSTATIN AT 50° C.

Time, wks.	Colorimetric Assay (u./mg.) Control, 0°	Stability, 50°	Per cent of Control[b]	Microbiological Assay (u./mg.)[a] Control, 0°	Stability, 50°	Per cent of Control[c]
0	3504	...	...	3360	...	...
3	...	3044	86.7	3290	2820	85.7
5	...	2914	83.0	3300	2770	83.9
7	3504	2740	78.1	3170	2380	75.1
9	3524	2673	76.2	3020	2370	78.5
11	3533	2564	73.1	3270	2270	69.4
17	3480	2300	65.5	3030	2040	67.3
20	...	2384	67.9	2980	1940	65.1

[a] Assayed by the agar-diffusion method using *Saccharomyces cerevisiae*. [b] Calculated from the average of the assays of the control samples. [c] Calculated from the corresponding assay of the control sample.

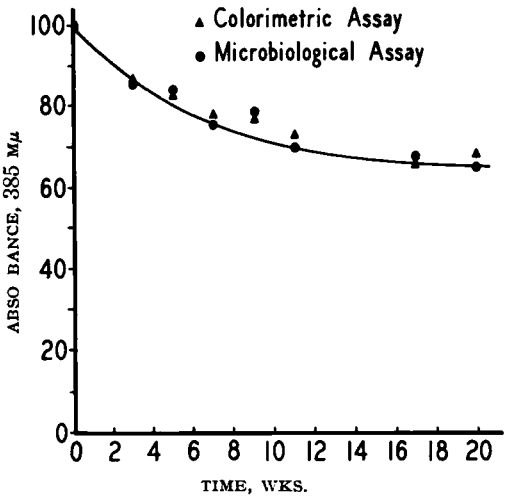

Fig. 2.—Degradation of nystatin at 50° C.

natant solution transferred into a 100-ml. volumetric flask. The volume was made up with dimethylformamide.

Ointment.—Same as *Cream*, but without centrifuging.

Capsule (containing tetracycline phosphate complex).—An amount of solids containing about 70,000 u. of nystatin from a mixture of the contents of five capsules was weighed into a 50-ml. centrifuge tube containing about 200 mg. of magnesium chloride hexahydrate. Twenty-five milliliters of water was added, and the tube shaken for 1 minute. After centrifuging, the water solution was discarded. The tube was washed with 25 ml. of water, centrifuged, and the water again discarded. The residue was then dissolved in small portions of dimethylformamide and transferred into a 100-ml. volumetric flask. The volume was made up with dimethylformamide.

All the nystatin solutions were prepared in low actinic glass volumetric flasks.

Degradation Study

One sample of powder (Powder 1) was chosen for a degradation study. Samples of 500 mg. each were placed in 2 × 9-cm. open vials spread out to a thickness of 0.5 cm. and stored in an oven at 50 ± 2°. The samples were withdrawn periodically over 20 weeks and assayed by both the colorimetric and microbiological methods.

RESULTS AND DISCUSSION

Results of assays of nystatin as the powder and in pharmaceutical preparations are shown in Table I along with comparisons to the microbiological assays and to the spectrophotometric assays (8) for four samples of powder. It can be seen that the agreement is good. The colorimetric assays show good reproducibility with a standard deviation of about 2%. The standard deviation for the microbiological assay is about 3.3% for the powders, and about 3.7% for the creams, ointments, and capsule.

Results of the degradation study are shown in Table II. A plot of the stability assays expressed as percentage of the control assay *vs.* time is shown in Fig. 2. Since the microbiological control assays varied quite widely due to differences in sensitivity of the organism, the percentages of the microbiological stability assays are calculated from the corresponding control assays. The percentages of the colorimetric stability assays, on the other hand, are calculated from the average of the control assays because deviation in the colorimetric control assays was small, the largest difference being only 1.5%. From the plot in Fig. 2, it can be seen that all the points lie closely on the same curve, demonstrating the colorimetric assay to be a stability assay.

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Pyridazines I

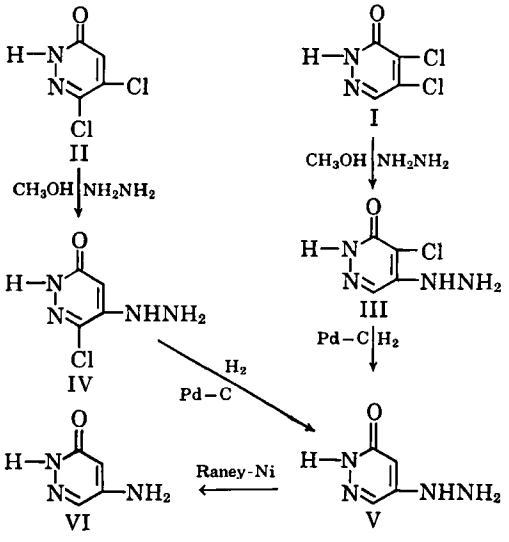
4-Halo-5-hydrazino-3-pyridazone and Its Derivatives

By WINNIFRED M. OSNER, RAYMOND N. CASTLE, and DUANE L. ALDOUS

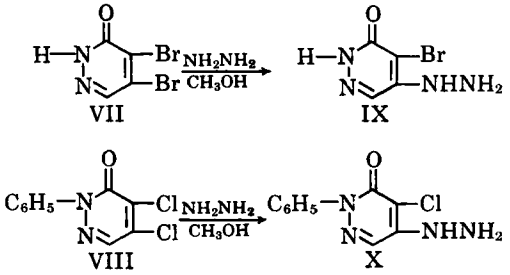
When 4,5-dichloro-3-pyridazone, 4,5-dibromo-3-pyridazone, or 4,5-dichloro-2-phenyl-3-pyridazone in methanol was allowed to react with 95% hydrazine, a mono-halo mono-hydrazino-3-pyridazone was obtained. An unequivocal proof of structure for 4-chloro-5-hydrazino-3-pyridazone is reported. Twenty-five hydrazones of 4-bromo-5-hydrazine-3-pyridazone and twenty-seven hydrazones of 4-chloro-5-hydrazino-3-pyridazone were prepared. One cyclized derivative of 4-chloro-5-hydrazino-2-phenyl-3-pyridazone is also reported.

During investigations in this laboratory of the relative reactivity of the chlorine atoms of 4,5-dichloro-3-pyridazone (I), the very facile reaction of this compound with hydrazine to yield a mono-halo mono-hydrazino-3-pyridazone was noted. The structure of 4-chloro-5-hydrazino-3-pyridazone (III) was determined by catalytic dechlorination followed by Raney-Ni cleavage of the hydrazino group to give the known 5-amino-3-pyridazone (VI) first prepared by Kuraishi (1).

Further confirmation was obtained by allowing 5,6-dichloro-3-pyridazone (II) to react with hydrazine. The product, compound IV, was catalytically dechlorinated to produce a compound (V) identical in all respects to that obtained from 4,5-dichloro-3-pyridazone under the same conditions.



4,5-Dibromo-3-pyridazone (VII) was also treated with hydrazine as was 4,5-dichloro-2-phenyl-3-pyridazone (VIII) and structures for the products—compounds IX and X, respectively—were assigned by analogy. The properties of compounds III through VI, IX, and X are listed in Table I.



The pyridazines have long been the subject of pharmacological studies, and the activities attributed to compounds of this ring system vary greatly (2–5). Similarly, the hydrazino group has frequently been found to confer activity upon a given structure. A complete review of hydrazine derivatives used as medicinals was published by Jucker in 1959 (6). With these facts in mind, it was deemed appropriate to prepare several carbonyl derivatives of both the bromo and chloro hydrazino compounds.

The properties of the derivatives are listed in Table II. Where a 1,3- or 1,4-dicarbonyl compound was employed, a cyclized derivative was obtained. Structures and properties for these products are given in Table III.

Infrared and ultraviolet absorption spectra of all the compounds prepared were recorded and contribute confirmatory evidence for the structure assigned.[1]

Data have been received concerning 23 of the compounds submitted to Cancer Chemotherapy National Service Center, National Cancer Insti-

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[1] The spectra appear in the doctoral dissertation by Winnifred M. Osner. Copies are available through University Microfilm, Inc., 313 N. First St., Ann Arbor, Mich.

TABLE I.—HYDRAZINO AND AMINO-3-PYRIDAZONES

Compound	M.p., °C.	Formula	Analysis C Calcd.	C Found	H Calcd.	H Found
III	195 dec.	$C_4H_5N_4OCl$	29.92	30.27	3.14	3.22
IV	268 dec.	$C_4H_5N_4OCl$	29.92	30.29	3.14	3.19
V	259 dec.	$C_4H_6N_4O$	38.09	38.16	4.79	4.59
VI	289 dec.	$C_4H_5N_3O$	43.24	42.94	4.54	4.10
IX	180 dec.	$C_4H_5N_4OBr$[a]	23.43	23.78	2.46	2.14
X	164 dec.	$C_{10}H_9N_4OCl$[a]	50.75	50.47	3.83	3.51

[a] Inactive in first stage of screening.

tute, for tissue culture screening. Though it is in no way complete or conclusive, this information as indicated in the tables is included here for consideration.

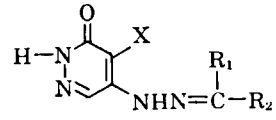
EXPERIMENTAL

All melting points were determined with a Vanderkamp Melt-Pointer and are uncorrected.

4 - Chloro - 5 - hydrazino - 3 - pyridazone (III).—Thirty-three Gm. (0.2 mole) of 4,5-dichloro-3-pyridazone (I) prepared by the method of Mowry (7) were dissolved in 560 ml. of boiling methanol. To this solution 19 Gm. (0.6 mole) of 95% hydrazine was added portionwise, and a yellow precipitate appeared after 10 minutes. The mixture was allowed to reflux a total of 1.5 hours; then it was cooled and filtered. Recrystallization from water afforded 20 Gm. (62%) of pale yellow needles, m.p. 195° dec.

4 - Bromo - 5 - hydrazino - 3 - pyridazone (IX).—4,5-Dibromo-3-pyridazone prepared by the method

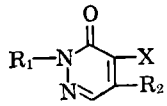
TABLE II.—DERIVATIVES OF 4-HALO-5-HYDRAZINO-3-PYRIDAZONE

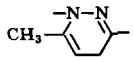
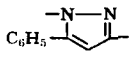
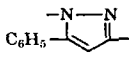
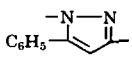


X	R_1	R_2	M.p., °C.	Formula	Analysis C Calcd.	C Found	H Calcd.	H Found	Prepn.
Br	Hydrogen	Phenyl	241 dec.	$C_{11}H_9N_4OBr$[b]	45.07	44.93	3.09	3.01	A
Cl	Hydrogen	Phenyl	304 dec.	$C_{11}H_9N_4OCl$[b]	53.11	53.30	3.65	3.57	A
Br	Hydrogen	*m*-Hydroxyphenyl	267 dec.	$C_{11}H_9N_4O_2Br$	42.74	42.49	2.93	2.86	A
Cl	Hydrogen	*m*-Hydroxyphenyl	300 dec.	$C_{11}H_9N_4O_2Cl$	49.91	49.73	3.42	3.21	A
Br	Hydrogen	3,4-Dimethoxyphenyl	248 dec.	$C_{13}H_{13}N_4O_3Br$	44.20	44.19	3.71	3.29	A
Cl	Hydrogen	3,4-Dimethoxyphenyl	276 dec.	$C_{13}H_{13}N_4O_3Cl$[c]	50.57	50.55	4.24	4.11	A
Br	Methyl	Phenyl	220 dec.	$C_{12}H_{11}N_4OBr$[c]	46.92	46.96	3.61	3.40	A
Cl	Methyl	Phenyl	255 dec.	$C_{12}H_{11}N_4OCl$[d]	54.88	54.66	4.22	3.99	A
Br	Methyl	*p*-Methylphenyl	224 dec.	$C_{13}H_{13}N_4OBr$	48.61	48.70	4.08	3.92	A
Cl	Methyl	*p*-Methylphenyl	280 dec.	$C_{13}H_{13}N_4OCl$	56.42	56.29	4.73	4.64	A
Br	Methyl	3,4-Dimethylphenyl	220 dec.	$C_{14}H_{15}N_4OBr$	50.16	50.29	4.51	4.44	A
Cl	Methyl	3,4-Dimethylphenyl	263 dec.	$C_{14}H_{15}N_4OCl$	57.83	57.81	5.20	4.78	A
Br	Methyl	*o*-Hydroxyphenyl	234 dec.	$C_{12}H_{11}N_4OBr$	44.60	44.79	3.43	3.50	A
Cl	Methyl	*o*-Hydroxyphenyl	289 dec.	$C_{12}H_{11}N_4O_2Cl$	51.71	51.58	3.98	3.84	A
Br	Methyl	3,4-Dichlorophenyl	240 dec.	$C_{12}H_9N_4OBrCl_2$	38.32	38.72	2.41	2.41	A
Cl	Methyl	3,4-Dichlorophenyl	314 dec.	$C_{12}H_9N_4OCl_3$	43.46	43.70	2.74	2.68	A
Br	Ethyl	Phenyl	182	$C_{13}H_{13}N_4OBr$	48.61	48.67	4.03	3.83	A
Cl	Ethyl	Phenyl	209–210	$C_{13}H_{13}N_4OCl$[b]	56.43	56.38	4.74	4.49	A
Br	*n*-Propyl	Phenyl	175–176	$C_{14}H_{15}N_4OBr$	50.16	50.14	4.51	4.21	A
Cl	*n*-Propyl	Phenyl	217–218	$C_{14}H_{15}N_4OCl$[a]	57.84	57.57	5.20	4.90	A
Br	Isopropyl	Phenyl	221 dec.	$C_{14}H_{15}N_4OBr$[a]	50.16	49.75	4.51	4.25	A
Cl	Isopropyl	Phenyl	251	$C_{14}H_{15}N_4OCl$[c]	57.83	57.56	5.20	4.95	A
Br	*n*-Butyl	Phenyl	151	$C_{15}H_{17}N_4OBr$	52.90	52.72	5.27	4.97	A
Cl	*n*-Butyl	Phenyl	190	$C_{15}H_{17}N_4OCl$	60.27	60.15	6.01	5.68	A
Br	2-Carboxyvinyl	Phenyl	233 dec.	$C_{14}H_{11}N_4O_3Br$[a]	46.30	46.36	3.05	2.78	A
Cl	2-Carboxyvinyl	Phenyl	255 dec.	$C_{14}H_{11}N_4O_3Cl$[c]	52.76	52.62	3.47	3.44	A
Br	Methyl	Styryl	207 dec.	$C_{14}H_{13}N_4OBr$	50.46	51.02	3.93	3.85	A
Cl	Methyl	Styryl	214 dec.	$C_{14}H_{13}N_4OCl$	58.34	58.96	4.54	4.47	A
Br	Phenyl	Phenyl	299 dec.	$C_{17}H_{13}N_4OBr$	55.30	55.46	3.54	3.32	B
Cl	Phenyl	Phenyl	304 dec.	$C_{17}H_{13}N_4OCl$	62.87	62.87	4.03	4.02	B
Br	Phenyl	*p*-Methoxyphenyl	232	$C_{18}H_{15}N_4O_2Br$	54.15	54.23	3.79	3.52	A
Cl	Phenyl	*p*-Methoxyphenyl	252	$C_{18}H_{15}N_4O_2Cl$	61.11	61.11	4.27	4.29	A
Br	Benzyl	*p*-Methylphenyl	236 dec.	$C_{19}H_{17}N_4OBr$[a]	57.44	57.25	4.31	4.09	A
Cl	Benzyl	*p*-Methylphenyl	276 dec.	$C_{19}H_{17}N_4OCl$	64.68	64.41	4.86	4.73	A
Br	Benzyl	Benzyl	192 dec.	$C_{19}H_{17}N_4OBr$[a]	57.44	57.33	4.31	4.30	A
Cl	Benzyl	Benzyl	207	$C_{19}H_{17}N_4OCl$	64.68	64.53	4.86	4.41	A
Br	Phenyl	Phenylhydroxymethyl	240 dec.	$C_{18}H_{15}N_4O_2Br$	54.15	53.69	3.79	3.31	A
Cl	Phenyl	Phenylhydroxymethyl	259 dec.	$C_{18}H_{15}N_4O_2Cl$	60.93	60.81	4.26	3.93	A
Br	Phenyl	Benzoyl	225	$C_{18}H_{13}N_4O_2Br$	54.42	54.11	3.30	3.06	A
Cl	Phenyl	Benzoyl	220 dec.	$C_{18}H_{13}N_4O_2Cl$[d]	61.28	61.43	3.71	3.55	A
Cl	Hydrogen	2-Furyl	259 dec.	$C_9H_7N_4O_2Cl$[b]	45.30	45.05	2.96	2.78	A
Br	Methyl	3-Pyridyl	267 dec.	$C_{11}H_{10}N_5OBr$[a]	42.87	43.05	3.27	3.19	B
Cl	Methyl	3-Pyridyl	280 dec.	$C_{11}H_{10}N_5OCl$	50.10	49.80	3.82	3.69	A
Br	Methyl	2-Pyridyl	251 dec.	$C_{11}H_{10}N_5OBr$[a]	42.87	42.81	3.27	3.18	B
Cl	Hydrogen	3-Pyridyl	287 dec.	$C_{10}H_8N_5OCl$	48.11	47.63	3.23	3.11	A
Br	Phenyl	*p*-Dimethylaminophenyl	213 dec.	$C_{19}H_{18}N_5OBr$	55.35	55.34	4.40	4.66	A
Cl	Phenyl	*p*-Dimethylaminophenyl	258 dec.	$C_{19}H_{18}N_5OCl$	62.04	61.53	4.93	4.68	A
Cl	*p*-Dimethylaminophenyl	*p*-Dimethylaminophenyl	253 dec.	$C_{21}H_{23}N_6OCl$[c]	61.38	61.10	5.64	5.18	A
Cl	Hydrogen	*p*-Dimethylaminophenyl	252 dec.	$C_{13}H_{14}N_5OCl$	53.52	53.54	4.84	4.64	A

[a] Inactive in first stage of screening. [b] Active in first stage; inactive in second stage. [c] Active in first stage; no data on second stage. [d] Active through first and second stages.

TABLE III.—CYCLIC DERIVATIVES OF 4-HALO-5-HYDRAZINO-3-PYRIDAZONE



X	R_1	R_2	M.p., °C.	Formula	Analysis C Calcd.	C Found	H Calcd.	H Found	Prepn.
Br	Hydrogen		264 dec.	$C_{10}H_{11}N_4OBr$[a]	42.42	42.49	3.92	3.39	A
Br	Hydrogen		211–213	$C_{19}H_{13}N_4OBr$	58.03	58.14	3.33	2.99	A
Cl	Hydrogen		209	$C_{19}H_{13}N_4OCl$[b]	65.44	65.51	3.76	3.64	A
Cl	Phenyl		176–178	$C_{25}H_{17}N_4OCl$	70.67	70.42	4.03	3.80	A

[a] Active in first stage; inactive in second stage. [b] Active in first stage; no data on second stage.

of Bistrzycki (9) was treated with hydrazine as described above for compound III. Recrystallization from water produced dark yellow needles, m.p. 180° dec.

4-Chloro-5-hydrazino-2-phenyl-3-pyridazone (X)—4,5-Dichloro-2-phenyl-3-pyridazone (VIII) prepared by the method of Mowry (7), when allowed to react with hydrazine as described above, gave a 75% yield of fine yellow needles, m.p. 164° dec.

6-Chloro-5-hydrazino-3-pyridazone (IV).—5,6-Dichloro-3-pyridazone (II), prepared by the method of Kuraishi (8), was similarly treated with hydrazine. The product (94%) was recrystallized from water to yield white crystals, m.p. 268° dec.

5-Hydrazino-3-pyridazone (V).—4-Chloro-5-hydrazino-3-pyridazone (III) (4.8 Gm., 0.05 mole) dissolved in 200 ml. of 1% sodium hydroxide was treated with hydrogen in the presence of 1.2 Gm. of 5% Pd-C catalyst at atmospheric pressure and room temperature. Following absorption of the theoretical amount of hydrogen, the catalyst was separated and washed with 50 ml. of 1% sodium hydroxide followed by methanol until washings were no longer basic. The filtrate and washings were neutralized with glacial acetic acid and the solution concentrated on a steam bath under reduced pressure. After cooling 48 hours, 2.8 Gm. (74%) of a dark orange solid was collected. Recrystallization from 95% ethanol (norite) afforded pale yellow needles, m.p. 267° dec.

5-Amino-3-pyridazone (VI).—A mixture of 1.75 Gm. (0.014 mole) of 5-hydrazino-3-pyridazone (V) dissolved in 85% ethanol and Raney-Ni W-2 (prepared from 15 Gm. of Raney-Ni alloy) was allowed to reflux 2 hours. The catalyst was removed and the filtrate evaporated on a steam bath under reduced pressure to approximately one-half volume. After cooling overnight, 0.9 Gm. (60%) of gray plates was collected and recrystallized from water (Norite) to yield white crystals, m.p. 288° dec.

General Procedure A.—One-tenth mole of the appropriate halo-hydrazino-pyridazone was treated with 8 ml. concentrated sulfuric acid followed by 70 ml. water and the mixture heated gently just until the solid dissolved, at which time the hot solution was poured into a second flask containing 1 ml. of carbonyl compound dissolved in 40–80 ml. 95% ethanol. After standing 12 to 48 hours, the solid was filtered and recrystallized usually from 95% ethanol.

General Procedure B.—To one-tenth mole of the halo-hydrazino-pyridazone were added 10 ml. concentrated hydrochloric acid in 10 ml. water and 15 ml. 95% ethanol. The mixture was heated then added to 1 Gm. of the carbonyl compound dissolved in 10 ml. of ethanol. After cooling 24 hours, the precipitate was filtered and recrystallized from an appropriate solvent.

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Substituted Salicylanilides III

New Salicylanilides and Related Compounds with Antimicrobial Activity

By ROBERT G. TABORSKY† and ROLAND J. STARKEY

Eighteen substituted salicylanilides and related compounds have been synthesized and screened for antimicrobial activity. Most of these compounds have been prepared for the first time. The relationship of structure and activity for the series is discussed.

INTEREST IN SUBSTITUTED salicylanilides was a result of the observation of a high degree of antimicrobial activity of halonitrosalicylanilides (1) and other substituted salicylanilides (2). The first objective of the present investigation was to attempt to define further the structural requirements necessary for antimicrobial activity for this series. Some of the previously prepared salicylanilides possessed limitations to their usefulness because of toxicity, solubility, color, and inactivation by blood serum. Thus the second objective was to see whether new and more favorable members of the series could be developed.

All of the compounds under discussion are summarized and described in Table I. A considerable group (I–VIII) of those salicylanilides were synthesized by the reaction of a suitably substituted salicoyl chloride with an amine in an appropriate organic solvent, usually benzene.

Salicylanilides substituted in the "three" or "five" positions by an amino or acetylamino group were prepared by reduction of the respective nitro analogs. The reducing agent used for the former was sodium dithionate (3), and for the latter it was zinc and glacial acetic acid. The amino compound was converted to the diazo derivative by a normal diazotization (3).

The phthalyl-mono-chloroanilides were prepared by the reaction of a substituted or unsubstituted phthalic anhydride with *para*-chloroaniline (4). The imine, N-(5-nitrosalicylidene) *meta*-chloroaniline was prepared by the condensation of 5-nitrosalicylaldehyde and *para*-chloroaniline in ethanol.

4′ - Chloro - 5 - (N - *para* - chlorophenyl - sulfamido)salicylanilide was prepared by refluxing together 5-sulfosalicylic acid and *para*-chloroaniline with phosphorus trichloride in chloroform. 4′-Chloro-5-sulfamidosalicylanilide was prepared in a similar manner from 5-sulfamidosalicylic acid and *para*-chloroaniline.

All of the compounds have been synthesized for the first time with the exception of III, XI, XII, and XIII. The compounds were screened against two significant organisms by the cup-plate method. The results of that primary screening are summarized in Table II.

In the ensuing discussion, it must be remembered that all of the antimicrobial screening data are qualitative. The philosophy of the investigation has been to prepare compounds where certain parts of the previously prepared active halonitrosalicylanilides have been replaced by other groups, holding the remaining portions of the molecule unchanged. Therefore, in the following discussion, the halonitrosalicylanilides are viewed somewhat as parent compounds.

Several generalizations can be made from the results in Table II. In halonitrosalicylanilides where the *para*-chloroaniline moiety has been replaced by morpholine, picoline, or *meta*-carboxyaniline (*meta*-aminobenzoic acid), all activity was lost. Since 3′-chloro-5-nitrosalicylanilide has previously been shown to be active (1), it is apparent that a carboxyl group cannot replace a chlorine atom. On the other hand, the activity of III does show that a trifluoromethyl group can replace a chlorine atom with retention of activity. Furthermore, activity was retained when an aminothiazole entity replaced the *para*-chloroaniline portion of the molecule.

A degree of lipid solubility has at times been postulated as requisite for antimicrobial or drug activity (5, 6) in order to enable the molecule to cross or else combine with the lipid portion of the cell membrane. The halogen or nitro groups needed as substituents on salicylanilide to make it generally microbicidal probably increases its lipid solubility.

Since 4′-bromo-5-bromosalicylanilide is a known antimicrobial (7), 4′-dimethylamino-5-bromosalicylanilide (VII) was prepared with the idea that the dimethylamino group would confer

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TABLE I.—SUBSTITUTED SALICYLANILIDES AND RELATED COMPOUNDS

No.	Compound	Formula	Anal. Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N	M.p., °C. (uncor.)	Yield, %
I	5-Nitrosalicylmorpholide	$C_{11}H_{12}N_2O_5$	52.3	4.8	11.1	52.7	4.8	10.9	241–244	98
II	5-Nitrosalicylpiperide	$C_{12}H_{14}N_2O_4$	57.5	5.6	11.2	57.7	5.5	11.3	245–246	99
III	3′-Trifluoromethyl-5-nitrosalicylanilide	$C_{14}H_9F_3N_2O_4$	51.5	2.8	8.6	51.6	2.9	8.7	180–182.5 (Reported, 182 (11))	86
IV	3′-Carboxy-5-nitrosalicylanilide	$C_{14}H_{10}N_2O_6$	55.6	3.3	9.3	55.3	3.6	9.2	316	67
V	N-Methyl-5-nitrosalicylanilide	$C_{14}H_{12}N_2O_4$	61.9	4.4	10.3	61.4	4.4	10.0	135–137	..
VI	N-2′-Thiazolyl-5-nitrosalicylamide	$C_{10}H_7N_3O_4S$	45.4	2.3	15.9	45.4	2.6	15.5	289[a]	..
VII	4′-Dimethylamino-5-bromosalicylanilide	$C_{15}H_{15}BrN_2O_2$	53.7	4.5	8.4	53.9	4.7	7.4	226	100
VIII	4′-Dimethylamino-5-bromosalicylanilide hydrochloride	$C_{15}H_{16}ClBrN_2O_2$	Chlorine, 9.4	..	...	...	Chlorine, 8.8	...	223[a]	100
IX	4′-Chloro-5-acetylaminosalicylanilide	$C_{15}H_{13}ClN_2O_3$	59.5	4.2	9.0	59.7	4.4	8.8	280	81
X	4′-Chloro-3-acetylaminosalicylanilide	$C_{15}H_{13}ClN_2O_3$	59.5	4.2	9.0	58.5	4.3	8.9	216–218	81
XI	4′-Chloro-5-aminosalicylanilide	$C_{13}H_{11}ClN_2O$	...	..	...	...	..	...	200–203 (Reported, 208–210 (3))	92
XII	4′-Chloro-5-diazosalicylanilide	$C_{13}H_9ClN_2O$	...	..	...	...	..	...	151[a] (Reported, 143[a] (3))	78
XIII	4′-Chloro-5-mono-phthalylanilide	$C_{14}H_{10}ClNO_3$	...	..	...	...	..	...	182–183 (Reported, 183–184 (4))	100
XIV	4′-Chloro-mono-3-nitrophthalylanilide	$C_{14}H_9ClN_2O_5$	52.5	2.8	8.8	52.0	3.0	8.1	183–185	100
XV	N-(5-Nitrosalicylidene)3′-chloroaniline	$C_{13}H_9ClN_2O_3$	56.5	3.3	10.2	56.0	3.1	10.0	205–206	79
XVI	4′-Chloro-5-(N-*para*-chlorophenylsulfamido) salicylanilide (monohydrate)	$C_{19}H_{14}Cl_2N_2O_5S$	50.3	3.1	6.2	50.1	3.7	6.3	250–251	73
XVII	4′-Chloro-5-sulfamidosalicylanilide	$C_{13}H_{11}ClN_2O_4S$	50.0	3.5	4.5	50.2	3.5	4.9	234–237	72
XVIII	5-(N-*para*-Chlorophenylsulfamido) salicylic acid	$C_{13}H_{10}NO_5S$	48.0	3.7	4.3	47.9	3.1	4.0	228	92

[a] Melted with decomposition.

equivalent or greater lipid solubility as the bromine atom to the molecule. However, the results were disappointing because the compound and its hydrochloride were both completely inactive. The hydrochloride was water insoluble which indicated that probably the dimethylamino group did increase lipid solubility. However, solubility versatility in both lipid and water may possibly be more important than either extreme as was shown to be the case for 8-hydroxyquinoline (oxine) (5).

Attempts to replace the nitro group of the active halonitrosalicylanilides by a sulfamido (XVII) or N-*para*-chlorophenylsulfamido group (XVI) produced compounds lacking antimicrobial activity. When an imine linkage replaced the amide linkage of 3′-chloro-5-nitrosalicylanilide, the resultant anil retained activity.

Recently, there has appeared in the literature an attempted correlation of pKa and activity for several salicylanilides with the idea that greater pKa represents greater chelating potential, which is possibly important to activity (8). For this reason and because of their ready synthesis, two phthalyl-mono-*para*-chloroanilides were prepared (XIII and XIV). In essence, compound XIV is equivalent to 4′-chloro-5-nitrosalicylanilide where, however, a carboxyl group has replaced the *ortho*-hydroxyl radical. However, such compounds were completely without antimicrobial activity. Although the acidity of the *ortho* group has been increased, the hydroxyl portion is farther in space and its connections to the ring lack the rigidity of the hydroxyl. Further, it would have to form a seven-membered ring with a chelating metal (hydroxyl-metal-carbonyl oxygen). Therefore, the hydroxyl group on the carboxyl would have a higher spatial entropy content (less ordered) and would require greater energies to enter into a chelate bond which probably would not be sufficiently offset by the stronger chelate bond of the ionic portion of the chelate ring due to a higher pKa. The inactivity of these latter compounds, therefore, does not necessarily rule out chelation as a factor for the active members.

A nitrosalicylanilide (V) was prepared having the amide nitrogen methylated with the resulting compound being inactive. A similar observation was made by

TABLE II.—ANTIMICROBIAL ACTIVITY OF SOME SUBSTITUTED SALICYLANILIDES AND RELATED COMPOUNDS

Compound No.	Antimicrobial Activity[a] *Staphylococcus aureus*[b]	*Epidermophyton floccosum*[c]
I	0	0
II	0	0
III	16	4
IV	0	0
V	0	0
VI	12	1
VII	0	0
VIII	0	0
IX	9	3
X	8	1
XI	2	0
XII	7	2 soln.
	4	24 solid
XIII	0	1
XIV	0	0
XV	8	5
XVI	0	0
XVII	0	0
XVIII	0	0

[a] Activity was determined by the agar cup-plate technique. The width of the zones of inhibition are expressed in mm. with no activity being represented by 0. [b] ATCC No. 6438. [c] ATCC No. 10227.

other investigators with the halogenated salicylanilides (2). In that article some of the requirements for activity among these compounds were discussed.

As a result of the present study, it has been possible to broaden some of the past known requisites for activity in the series. Of the compounds prepared that were active but did not possess a nitro group were those that yet retained a nitrogen on the "five" or "three" positions, in an amino (XI), acetylamino (IX, X), or diazo (XII) group. These latter compounds seem worthy of further investigation since they may possess physical and biological characteristics superior to other salicylanilides previously prepared.

EXPERIMENTAL[1]

Reactions of 5-Nitrosalicoyl Chloride and Amines.—Compounds I through VI were prepared by adding a benzene solution of 5-nitrosalicoyl chloride (1) to organic solutions of 2.5 molar excesses of morpholine, piperidine, *meta*-trifluoromethylaniline, *meta*-aminobenzoic acid, N-methylaniline, and 2-aminothiazole, respectively. VII was prepared from 5-bromosalicoyl chloride and N,N-dimethylphenylene diamine.

The amines were dissolved into benzene with the exception of *meta*-aminobenzoic acid, where diglyme and dimethylformamide were used as solvents. In each case the reaction mixture was vigorously stirred during the admixture and then allowed to stand at room temperature overnight. In each case a precipitate formed (usually very thick) consisting of the amine hydrochloride or a mixture of it with the respective product. The solids were filtered and varying amounts of further product could then be recovered by evaporating the filtrate to dryness. A preliminary purification was carried out by washing the product with 10% aqueous hydrochloric acid which removed the amine hydrochloride and amine. The treated solid was then dried and crystallized from boiling ethanol, sometimes adding either water or dimethylformamide to modify its solvency as necessary. The yields ranged from 67 to 100%.

Preparation of 4'-Chloro-3-acetylamino- and 5-Acetylaminosalicylanilide.—Both of these compounds were prepared in identical fashion which can be exemplified by the method for 4'-chloro-3-acetylaminosalicylanilide.

A solution of 5 Gm. (0.02 mole) of 4'-chloro-3-nitrosalicylanilide in 50 ml. of glacial acetic acid was heated to 85° and 20 Gm. of zinc dust was slowly added; vigorous action resulted. The mixture was then refluxed for 16 hours and vacuum filtered while hot to remove the zinc. The zinc was washed on the Büchner with small portions of boiling glacial acetic acid, the washings were added to the filtrate. Upon cooling the filtrate 4 hours at 5°, 5.3 Gm. (86.3% yield) of 4'-chloro-3-acetylaminosalicylanilide was obtained from the cooled solution upon filtration, m.p. 216–220°. Crystallization from chloroform gave a white product, m.p. 220–220.5°.

In a similar fashion, 5.0 Gm. of 4'-chloro-5-nitrosalicylanilide yielded 3.1 Gm. of 4'-chloro-5-acetylaminosalicylanilide which crystallized from alcohol as a white solid, m.p. 280–284°.

Preparation of 4'-Chloro-5-aminosalicylanilide and the Respective 5-Diazo Derivative.—This compound was prepared according to a recently reported procedure of reducing 4'-chloro-5-nitrosalicylanilide with sodium dithionate (3). It was then treated with nitrous acid according to the same reference to yield 4'-chloro-5-diazosalicylanilide.

Preparation of N-(5-Nitrosalicylidene) *meta*-Chloroaniline.—Two grams (0.012 mole) of 5-nitrosalicylaldehyde in 35.0 ml. of ethanol was added to 2.0 ml. (2.4 Gm.) (0.02 mole) of *meta*-chloroaniline in 10 ml. of ethanol. An immediate precipitate formed that filled the flask. The mixture was filtered after 2 hours to obtain a quantitative yield (3.3 Gm.) of the product. Crystallization from ethanol-dimethylformamide yielded yellow crystals, m.p. 205–206°.

Preparation of Monoanilides of Phthalic Acid.—Phthalyl-mono-*para*-chloroanilide was prepared from phthalic anhydride and *para*-chloroaniline according to a method described in the literature (4). In a similar manner the 3-nitrophthalic acid analog was prepared.

Twenty-one grams (0.1 mole) of 3-nitrophthalic anhydride was suspended in 100 ml. of chloroform and 12.7 Gm. (0.1 mole) of *para*-chloroaniline was added. An immediate warming and deepening of color and very heavy precipitation occurred which solidified the mixture. More chloroform was added to bring the total volume to about 300 ml. The mixture was allowed to stand overnight at room temperature, vacuum filtered, washed with chloroform, and dried to give a quantitative yield (32.0 Gm.) of 3-nitrophthalyl-mono-*para*-chloroanilide, m.p. 183.5°.

[1] Antimicrobial screening was carried out by the Wisconsin Alumni Association.

Preparation of 5-Sulfamidosalicylic Acid and N-Substituted Derivatives.—5-Chlorosulfonylsalicylic acid was prepared by adding dried salicylic acid to chlorosulfonic acid (9). A purified product was obtained upon crystallization from benzene, m.p. 171–174° (reported m.p. 171–172° (9)).

5-Sulfamidosalicylic acid was prepared by reacting the above chloride with aqueous ammonium hydroxide. Isolation and crystallization yielded a product, m.p. 256–259°, (reported m.p. 253–255° (10)).

Preparation of 4′-Chloro-5-sulfamidosalicylanilide.—Six-hundred milligrams (0.003 mole) of 5-sulfamidosalicylic acid was ground in a pestle to an intimate mixture with 300 mg. (0.004 mole) of *para*-chloroaniline. The mixture was placed into 8.0 ml. of chloroform along with 0.4 ml. of phosphorus trichloride and refluxed 72 hours. Upon cooling and filtering, 880 mg. of mixed products were obtained. These were washed with a small amount of water and crystallized from 1:2 water-ethanol to obtain the product, m.p. 234–237°.

Preparation of 5-(N-*para*-Chlorophenylsulfamido)-salicylic Acid.—Anhydrous 5-sulfosalicylic acid was prepared by taking the monohydrate and drying at 100° under a vacuum of 3 mm. for 18 hours. Ten grams (0.08 mole) of *para*-chloroaniline, 10.0 Gm. (0.05 mole) of the above anhydrous 5-sulfosalicylic acid, 75.0 ml. of chloroform, and 5.0 ml. of phosphorus trichloride were refluxed for 3 days. The mixture was then cooled in ice for several hours and filtered to obtain 14.6 Gm. of dried product. A portion was suspended in water, filtered, and crystallized from ethanol. A bright yellow insoluble material was removed by filtration during this operation. The filtrate produced white crystals of the title compound (as a monohydrate) m.p. 250–251°.

Preparation of 4′-N,N-Dimethylamino-5-bromosalicylanilide.—Twenty grams (0.09 mole) of 5-bromosalicylic acid were refluxed overnight with 15 ml. of thionyl chloride, 0.1 Gm. of anhydrous aluminum chloride, and 80 ml. of benzene. The mixture was filtered and all of the solvents removed under vacuum. The residual 5-bromosalicoyl chloride was brought into solution with pure benzene. One-third of this benzene solution (0.03 mole) was added to 13.0 Gm. (0.1 mole) of *para*-N,N-dimethylphenylenediamine in 60 ml. of benzene. The mixture was shaken and allowed to stand overnight to yield 18.7 Gm. of a dark green product obtained by filtering and drying the reaction mixture.

A 5-Gm. portion of material was crystallized from 80 ml. of ethanol to yield 4.3 Gm. of a dark green product. A considerable amount of residue had been removed by filtration. The material was crystallized a second time from 45.0 ml. of 3:1 ethanol-dimethylformamide to yield a dark green crystalline 4′-dimethylamino-5-bromosalicylanilide, m.p. 226°.

A second 5-Gm. quantity of the original product was shaken with 50 ml. of water and 15.0 ml. of concentrated hydrochloric acid. This treatment considerably lightened the product from a dark green to white with a greenish cast. The 4.3 Gm. of product from this treatment was crystallized from 36 ml. of 1:1 ethanol-water to yield 3.8 Gm. of the hydrochloride of 4′-N,N-dimethylamino-5-bromosalicylanilide as light green granules, m.p. 223° (eff.).

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Effects of Ionizing Radiation on Two Gelatin Fractions II

Studies on Viscosity, Sedimentation Velocity, and Molecular Weight Effects

By LEONARD P. PRUSAK† and BARTLEY J. SCIARRONE

The effects of subdenaturant levels of irradiation on two fractions, F-I and F-II, isolated from commercial pigskin gelatin, were investigated through viscosity and sedimentation studies in 2*M* potassium thiocyanate solutions. The source of irradiation was a 3 Mev Van de Graaff accelerator. When used as controls, F-I and F-II were found to have intrinsic viscosity values of 0.675 and 0.550, respectively, while their respective average molecular weights were determined to be 173,000 and 86,000. After subjection to variable irradiation dose, the log of intrinsic viscosity values for F-I were directly proportional to the log of absorbed dose while an inverse proportionality occurred with F-II. Determination of Svedberg constants showed a similar pattern prevailed in the respective fractions when observed under sedimentation velocity conditions. Data obtained indicate random coil configuration was substantially undisturbed as radiation was applied, but bond rupture and linkage processes did occur.

IN OUR PREVIOUS PAPER (1) we described the isolation of the two fractions F-I and F-II from commercial pigskin gelatin by alcohol precipitation. We also discussed their acid/base titration behavior and the technique applied in irradiating these fractions in the form of 1% solutions, 5% gels, and dry films under the electron beam of a 3 Mev Van de Graaff Accelerator. We now report the results obtained in viscosity studies on both fractions before and after irradiation, their behavior under sedimentation velocity conditions, and the molecular weight determinations based on these data.

Viscosity measurements constitute a critical parameter in the evaluation of higher molecular weight polymers because of relationships to molecular weight, sedimentation rate, diffusion constant, and osmotic pressure.

The viscosity of a polymer solution (of which gelatin is a species) is a concentration-dependent factor. Huggins (2) established that the variation of viscosity with concentration of high polymer solutions can be expressed by

$$\frac{\eta_{sp}}{c} = [\eta] + k'[\eta]^2 c \qquad \text{(Eq. 1)}$$

where η_{sp}/c is the reduced viscosity, $[\eta]$ is referred to as the intrinsic viscosity and is defined as

$$\lim \left(\frac{\eta_{sp}}{c}\right) \text{ as } c \to 0$$

and k' is a polymer-solvent factor (also known as the Huggins constant or the hydrodynamic interaction constant).

The shape of the gelatin molecule in solution is generally accepted as being random coil. Boedtker and Doty (3) have so asserted on the basis of light scattering experiments. Williams, Saunders, and Cicirelli (4) established that the sedimentation rate constant for proteins in solution is proportional to the square root of the molecular weight; they concluded that gelatin molecules in solution exist in the form of a random coil. The equation of Staudinger in logarithm form appears to express best the relationship between intrinsic viscosity and molecular weight

$$\log [\eta] = \log K + \alpha \log M \qquad \text{(Eq. 2)}$$

where K is a solvent constant independent of molecular weight and α has a value dependent upon the size and shape of the molecule in question. For random coil gelatin Williams found the $\pm$ α value to be 0.62.

Pouradier and Venet (5), working with homogeneous fractions of Eastman Kodak F-74 gelatin (isoelectric point 4.75 and an average molecular weight 65,000), calculated α and K values through Eq. 2. Their values of 9.885 and 1.66×10^{-5} were for α and K, respectively.

The variable response of proteins to irradiation can be shown by their behavior under sedimentation velocity conditions. When considered together with intrinsic viscosity data, this information is useful in determining changes in molecular

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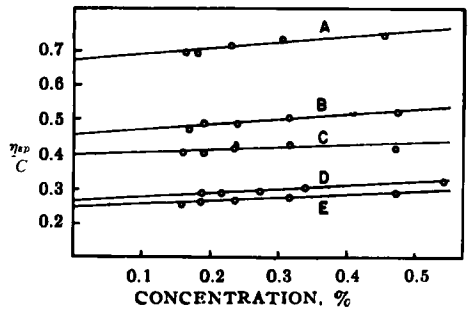

Fig. 1.—Plot of reduced viscosity *vs.* concentration to obtain intrinsic viscosity values for F-I before and after aqueous solutons were irradiated. One per cent solutions were irradiated (B = 0.17 Mrads.; C = 0.44 Mrads.; D = 1.27 Mrads., E = 2.80 Mrads.) and used as control (A). Viscosities were determined in 2 M KCNS solvent.

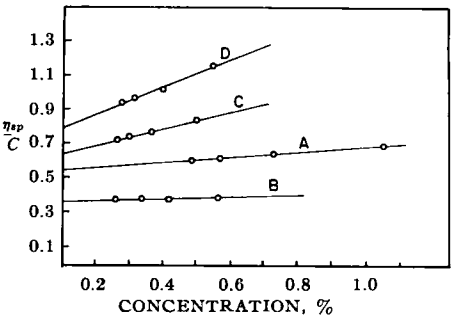

Fig. 2.—Plot of reduced viscosity *vs.* concentration to obtain intrinsic viscosity values for F-II before and after aqueous solutions were irradiated. One per cent solutions were irradiated (B = 0.16 Mrads.; C = 0.47 Mrads.; D = 1.19 Mrads.) and used as control (A). Viscosities were determined in 2M KCNS solvent.

weights. That such changes do occur has been shown by Alexander, *et al.* (6), who found that irradiation damage on solid crystalline bovine serum albumin (2 Mev electron beam) containing 4 to 6 per cent water (in the absence of oxygen) occurs in three stages. Primary ionization changes the shape of the molecule (as reflected in changes in sedimentation behavior) at doses insufficient to cause appreciable shifts in weight average molecular weights. This is the result of a breakdown of many secondary valency bonds, brought about by a single event which occurs on the average every 45 ev. A second ionization event opens the molecule still further so that half the disulfide bonds are accessible. Further irradiation causes extensive aggregation by intermolecular hydrogen bonds.

EXPERIMENTAL

Viscosity Studies.—Although considerable work has been reported on viscosity measurements of gelatin solutions, diverse opinion exists as to optimal solvent systems, pH, and temperature conditions. For this reason, preliminary runs were made under variable conditions to determine those most suitable for characterizing properly the particular fractions described here.

Water at 25° and 50° and sodium acetate-acetic acid buffer pH 4.8 at 25° and 50° gave unsatisfactory curve responses with respect to linearity and reproducibility. Sodium chloride 0.15 M at 25° and 50° gave positive straight-line plots with F-I and F-II but was not adopted as the experimental solvent because of its inability to inhibit aggregation (3) and to bring about reproducibility.

Straight-line plots of η_{sp}/c as a function of concentration were obtained with F-I and F-II in 2 M KCNS adjusted to pH 10.4. Viscosity determinations on irradiated 5% gels and dry films of F-I and F-II, after subsequent dilution to flowable 1% solutions, were conducted by pipetting a 5-ml. volume of the gelatin solution slowly into an Ubbelohde viscometer immersed in a water bath maintained at 25° ± 0.02°. An equal volume of 4 M KCNS was then added to the viscometer and (after gentle mixing) was allowed to come to temperature equilibrium. Subsequent dilutions were made with 5-ml. volumes of 2 M KCNS to maintain ionic strength constancy. Solvent flow time was 89.3 seconds. A sufficient number of readings were taken at each dilution level to assure reproducibility within ±0.05 second. A minimum of four dilutions followed the initial reading at 0.5% concentration. Solutions used in viscosity experiments were passed through a Seitz filter before irradiation.

TABLE I.—INTRINSIC VISCOSITY VALUES FOR CONTROL AND IRRADIATED FRACTIONS I AND II

F-I		F-II	
Dose, Mrads.	$[\eta]$	Dose, Mrads.	$[\eta]$
Control	0.675	Control	0.550
0.17	0.460	0.16	0.370
0.44	0.400	0.47	0.650
1.27	0.275	1.19	0.800
2.80	0.250	....	....

Reduced viscosity values plotted as a function of concentration for F-I and F-II are shown in Figs. 1 and 2. Intrinsic viscosity data are given in Table I. Log dose *vs.* log $[\eta]$ relationships were linear for both fractions; proportionality was inverse in the case of F-I and direct in the case of F-II.

The extrapolated $[\eta]$ value 0.675 obtained for F-I control corresponds closely to the 0.690 reported by Gouinlock, *et al.* (7), for their F-3 fraction, despite a 5° difference in temperature (30°) and half the molar concentration (1 M) of aqueous KCNS solvent. Pouradier's (5) value of 0.520 for his fraction Z1 (characterized as heterogeneous) is in reasonable agreement with our F-II, 0.550. The 0.640 value for his 5111 fraction is proximate to our F-I, 0.675.

Sedimentation Velocity Studies.—Rates of sedimentation of F-I and F-II in the form of 1% solutions as controls and irradiated in an aqueous environment were observed in the Spinco model E ultracentrifuge. Single and double sector cells were used on controls. Double sector cells were used exclusively on all irradiated samples. Sedimentation temperature was maintained at 25°, then corrected to 20°. In the case of controls run singly, instrument speed was set at 59,780 r.p.m. Double sector runs in all cases (except F-II irradiated at approximately

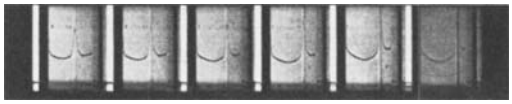

Fig. 3.—Sedimentation velocity run of 1% F-I in 2 *M* KCNS at 25°. Sedimentation boundary in a single sector cell was observed from right to left at 16-minute intervals using rotor speed of 59,780 r.p.m.

0.4 and 1.0 Megarad levels) were made at 39,460 r.p.m. Speeds in the excepted runs were reduced to 31,410 r.p.m. and 14,290–12,590 r.p.m., respectively, because of rapid boundary movement.

Figure 3 illustrates a sedimentation velocity run on F-I at 1% concentration in a single sector cell conducted at 59,780 r.p.m. at 25° in 2 *M* KCNS solvent. The sedimentation boundary was observed from right to left at 16-minute intervals. A similar peak was obtained at 0.7% concentration.

Single peaks were observed with F-I in double sector cells at 0.5% and 0.25% concentrations, at 39,460 r.p.m. observed at 32-minute intervals; single peaks with F-II at 0.9% and 0.45% concentrations, at 39,460 r.p.m. were observed at 16-minute intervals. Single peak characteristics prevailed in both fractions under all levels of irradiation employed.

Since irradiation was conducted on an aqueous system, sufficient quantities of potassium thiocyanate were subsequently added to each sample to bring the salt concentration to 2 *M* for sedimentation studies.

Sedimenting boundaries were analyzed with the aid of the Gaertner microcomparator. Plots of log x (where x is in cm. from axis of rotation) *vs.* t (in minutes) were constructed and extrapolated to zero time from which the uncorrected sedimentation coefficient was obtained through

$$S = \frac{dx/dt}{\omega^2 x} \qquad \text{(Eq. 3)}$$

Corrections of S to 20° were made through the relationship

$$S_{20w} = S_{obs}\left(\frac{\eta_t}{\eta_{20}}\right)\left(\frac{\eta}{\eta_0}\right)\left(\frac{1 - \bar{V}\rho_{20w}}{1 - \bar{V}\rho_t}\right) \qquad \text{(Eq. 4)}$$

where η_t is the viscosity of H_2O at 25° (8.949×10^{-3} poise); η_{20} is the viscosity of H_2O at 20° ($10\,087 \times 10^{-3}$ poise); η/η_0 is the factor for relative viscosity of 2 *M* KCNS to that of H_2O (0.915×10^{-2}); $\bar{V}$ is the partial specific volume of gelatin (0.695); ρ_{20w} is the density of the solvent (2 *M* KCNS) at 25° (1.090 Gm./ml.).

The ρ_t value was determined by experiment. Viscosity and density data for water are given by Svedberg (8). The specific volume factor and the η/η_0 relationship are those of Boedtker and Doty (9).

Plots of $1/S_{20w}$ *vs.* concentration were extrapolated to zero concentration and the reciprocal of the intercept taken as the coefficient value S^0 (Svedberg units). Results are shown in Table II and Figs. 4 and 5.

Molecular Weight Determinations.—Since the energy levels of irradiation imparted to F-I and F-II were designedly of low order, the extent of denaturation occurring in the irradiated solutions was small, and it was assumed that the random coil configura-

Table II.—Experimentally Determined Svedberg Constants (S^0) for F-I and F-II After Irradiation

F-I		F-II	
Sample Dose, Mrads.	S^0	Sample Dose, Mrads.	S^0
Control	4.25	Control	2.85
0.17	9.52	0.16	4.49
0.44	8.33	0.47	14.28
1.27	5.88	1.19	22.22
2.80	5.50		

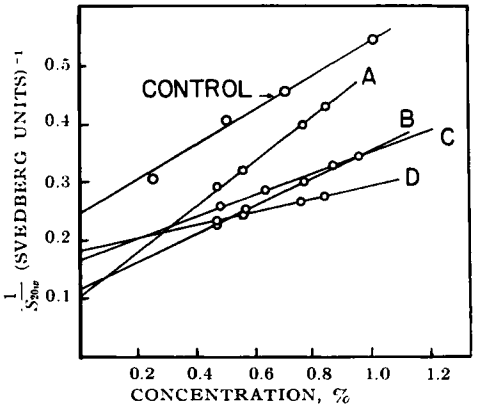

Fig. 4.—Determination of Svedberg constants by plotting the reciprocal of the corrected sedimentation coefficient of irradiated 1% aqueous solutions of F-I as a function of dilution in 2 *M* KCNS solvent. The doses imparted were: *A* = 0.17 Mrads., *B* = 0.44 Mrads., *C* = 1.27 Mrads., and *D* = 2.80 Mrads.

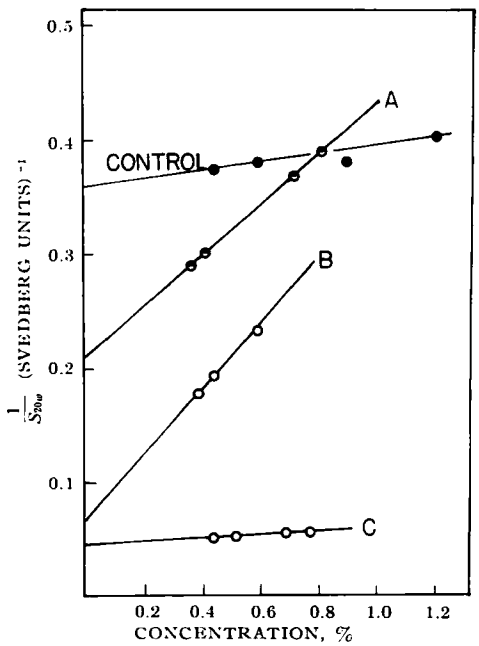

Fig. 5.—Determination of Svedberg constants by plotting the reciprocal of the corrected sedimentation coefficient of irradiated 1% aqueous solutions of F-II as a function of dilution in 2 *M* KCNS solvent. The doses imparted were: *A* = 0.16 Mrads., *B* = 0.47 Mrads., and *C* = 1.19 Mrads.

TABLE III.—MOLECULAR WEIGHT DATA AND α VALUES AS FUNCTIONS OF RADIATION DOSE

F-I		
Sample, Mrads.	Molecular Weight	α^a
Control	173,000	0.64
0.17	479,500	0.56
0.44	365,500	0.56
1.27	180,000	0.56
2.80	150,000	0.56
F-II		
Sample, Mrads.	Molecular Weight	α^a
Control	86,000	0.66
0.16	140,000	0.60
0.47	1,065,000	0.55
1.19	2,254,000	0.54

[a] From Eq. 2.

tion remained essentially intact. Intrinsic average molecular weights were calculated through the Scheraga-Mandelkern equation derived from the hydrodynamic elliposoidal model (10)

$$M = [\eta]^{1/2} \left(\frac{S^0 \eta_0 N}{\beta - (1 - \bar{V}\rho)} \right)^{3/2} \quad \text{(Eq. 5)}$$

A value of 0.915 for the viscosity of 2 *M* KCNS is in agreement with Boedtker and Doty (3). Taking 2.16×10^6 as the average beta value (11), molecular weights for F-I and F-II as well as their irradiated counterparts were obtained. The values are given in Table III along with α values determined from Eq. 2. A plot of the log of intrinsic viscosity *vs.* the log of molecular weight was linear for both fractions, as was the relationship of sedimentation constant to the square root of molecular weight, thus establishing maintenance of random coil configuration (4).

DISCUSSION

Viscosity Studies.—Since low order irradiation of F-I at 1% concentration produces successively lower intrinsic viscosity values in logarithmic relationship to dose, the question is posed whether scission plays a dominant role in reducing the viscosity of dilute gelatin solutions irradiated at these energy levels. According to Pasynsky (12) the rupture of peptide linkages, even if it does occur under the influence of biological radiation doses (0.3–2.5 R.), could not sufficiently change the configuration of the protein (serum albumin; gelatin) molecule. This, allegedly because the coiled state of the protein with numerous intramolecular bonds between chain links, is insensitive to radiation of this magnitude. As the dose is increased, internal structural changes take place, *i.e.*, aggregation, as reflected in an increase of absorption in the ultraviolet spectrum.

It is conceivable that these phenomena may occur in F-II, which shows progressive increase in viscosity after an initial decrease (with respect to the control). If the principle of proportionality between intrinsic viscosity and molecular weight is applied, one may assign responsibility for weight increases to (*a*) covalent linkage, or (*b*) excitation or activation of the molecules followed by regrouping the structural changes, *i.e.*, aggregation.

Bello, *et al.* (13), contend it is not likely that high intrinsic viscosities in unsolubilized gelatin are due to aggregation, but to crosslinking. Their experimental evidence, corroborated by the generally accepted capability of potassium thiocyanate to maintain gelatin solutions in a state of disaggregation, tends to negate the argument that aggregation is accountable for increased viscosity rather than crosslinking.

That large solvent:solute ratios are responsible for producing experimentally detectable intrinsic viscosity changes at low order irradiation levels is borne out by negligible and erratic viscosity changes in irradiated 5% solutions and films. Results obtained with films particularly give impetus to the proposition that secondary solvent-solute interactions during the ionization event are factors in producing shifts in intrinsic viscosity values. Impact of ionizing radiation on solid gelatin causes absorption of energy by the protein, but the absence of water during impingement deprives the molecules of freedom of movement and, consequently, interaction with solvent.

Wiederhorn, *et al.* (14), observed that thermally contracted (denatured) collagen obeys the kinetic theory of rubber elasticity. Assuming collagen to be a network polymer consisting of long chains tied together at intervals along their length, the average molecular weight of the chain between points of primary valence crosslinking was shown to be in the order of 55,000. This was based on the premise that the molecular weight between points of crosslinking can be determined from stress-strain behavior of a thermally contracted sample. From this quantity Wiederhorn deduced a method for calculating the number of crosslinks per unit volume of mass. The following mathematical relationship correlates the retractive force with elongation

$$f = \frac{v_2^{1/3}}{Mc} RT\rho \left(\alpha - \frac{1}{\alpha^2} \right) \quad \text{(Eq. 6)}$$

where f is the retractive force per unit cross section; α is the relative elongation of the sample, *i.e.*, the ratio of the stretch length to the initial length; ρ is the density of the unswollen sample; T is the absolute temperature; R is the gas constant 8.478×10^4 if the force is given in Gm./sq. cm. and the density in Gm./ml.; Mc is the average molecular weight of the chains between points of crosslinks; and v_2 is the volume fraction of the elastic constituent in the sample.

Employing a modified Wiederhorn device, stress-strain measurements were made on a series of commercial pigskin gelatin films irradiated as follows: control, 2.5, 5.0, 10.1, and 25.0 Megarads. It was found that the average molecular weight between crosslinks in the control varied from about 40,000–82,000; at the 2.5 Megarad level, it varied from 198,000–304,000, at the 5.0 Megarad level from 251,000–345,000, and at the 10 Megarad level from 499,000–1.2×10^6. Because of the brittleness of the sample and experimental difficulties, the stress-strain measurements on a 25.0 Megarad sample could not be obtained.

Determinations of molecular weight between crosslinks in irradiated F-I and F-II were unsuccessful. Films of both fractions irradiated at high (2.5 and greater Megarad) levels, succumbed to water at 60°, 65°, 75°, and 80°, indicating that thermostable crosslinking did not occur or, if it did, such crosslinking was of sufficiently low order to es-

cape Wiederhorn detection. Dry films (retained over calcium sulfate) and moist films (retained 12 hours over a water-saturated atmosphere), the latter theoretically providing the necessary electrons through an aqueous environment to enhance crosslinking, met with identical lack of success.

Sedimentation and Molecular Weight Studies.—It has been shown (4) that the relationship establishes random coil configuration of gelatin molecules in solution and entrapment of solvent within the coil where the sedimentation constant is a linear function of the square root of the molecular weight. On this basis, the proposition that irradiation at subdenaturant levels may have at least a partially scissive effect on the protein structure seems plausible. The decrease in molecular weight values (F-I) may be explained as due to a combination of moderate deaggregation and scission. The reverse effect (increase in molecular weight) would indicate a tendency toward crosslinking and weak bond formation. Further support for this suggestion is provided through linearly related sedimentation velocity *vs.* intrinsic viscosity and Svedberg constants (S^0) *vs.* the square root of molecular weight for both fractions.

One can now examine more closely the relationship between intrinsic viscosity and molecular weight as defined in the modified Staudinger equation (Eq. 2). The value of the semiempirical proportionality constant α is two for rigid rods, one for free-draining random coils and 0 for impenetrable spheres. Although independent of molecular weight, it is dependent on the flexibility of the polymer chain and solvent:solute characteristics. Assuming that the K factor of 2.9×10^{-4} is valid (4) for randomly coiled gelatin molecules, and that the exponential value α lies between 0.6–0.9 for a combination of the free-draining linear molecule and the swollen molecule with immobilized solvent, values of 0.64 for F-I and 0.66 for F-II are in reasonable agreement (Table III) with the established 0.62 value for randomly coiled gelatin. It appears justifiable to conclude that prior to irradiation both fractions had random coil convoluted configuration with solvent entrapment. In addition, it is evident from Table III that departure from the minimal 0.60 α value occurred on irradiation of both fractions.

It is interesting to note that Flory (15) sets an upper limit for α of 0.80 on the basis of randomly coiled linear polymer theory and a lower limit of 0.50. Since the constant is a function of solvent:solute interaction, the latter value would indicate the limit of a solvent capable of dissolving the polymer. The α values in all irradiated samples meet this requirement. It can be stated, therefore, that 2 M KCNS was capable of maintaining the gelatin fractions studied in a molecularly dispersed state and that random coil configuration was preserved.

A surprising result with respect to both fractions was the gross change in molecular weights imparted by small doses of energy on 1% solutions. This would seem contrary to prevailing opinion that low quanta of ionizing electrons produce relatively minor changes in protein (amino acid) structure. Although conceivably applicable in the case of solid states, it is not necessarily true of irradiated solutions. Considering that the higher molecular weight fraction (F-I) of the two studied nearly tripled in value on exposure to extremely low level irradiation and that (as the energy levels were increased) the molecular weight values became progressively lower, it is conceivable that the random coil configuration enters into a linking reaction, the extent of which diminishes in proportion to the intensity of irradiation. The lower molecular weight fraction (F-II) behaves conversely, *i.e.*, small quanta of radiation force linking of the protein. As the intensity of energy increases, so does the molecular weight, indicating direct proportionality between radiation and molecular weight. This behavior suggests that when gelatin molecules are subjected to excitation at high dilution they are oriented toward each other by forces induced through the mechanism of low order irradiation, causing a partial unfolding of the random coil, lengthening of the chain (as indicated in the initially high intrinsic viscosity value), and crosslinking. Provided that the energy imparted to the protein remains quantitatively below that required to rupture critical linkages, the attraction will induce an increase in molecular weight.

It seems reasonable to assume that the two fractions examined had random coil configuration with solvent entrapment and that an assignment of 0.6+ for the α value in the modified Staudinger equation is justified. Immobilization of the solvent within the swollen random coil can be inferred from the linear relationship between sedimentation constant and the square root of the molecular weight. Although F-I and F-II molecular weight values were related in 2:1 ratio, no experimental evidence was elicited which would establish substantial differences in the nature of the links holding the coiled entities together; the evidence suggested merely that a larger number prevailed. One can postulate as the most probable linkages van der Waals forces, weak hydrogen bonds (16), salt links, and covalent bonds joining the coils in a network structure. The lack of substantial coil bond strength is indicated by the lability toward denaturation by heat and, particularly in F-I and F-II, susceptibility to denaturation in aqueous solution on exposure to low order irradiation energy.

It is interesting to note that α decreases on irradiation of both gelatin fractions but does not in any case fall below 0.54. Evidently, application of stress to the freedraining random coil configuration effects increased coiling with consequent displacement of entrapped solvent as well as immobilization of solvent not displaced. This tendency follows the $[\eta]$ *vs.* $\sqrt{M_w}$ relationship already established.

Although (as noted previously) 2 M KCNS is acceptable as a solvent for maintaining gelatin in a molecularly dispersed state, some significance must be attached to the values obtained for k' in the modified Huggins equation (Eq. 1). This factor has been found to be a parameter of solute:solvent interaction. According to Thomas, *et al.* (17), for linear polymers in good solvents k' has values between 0.3–0.4. For F-I and F-II controls, F-I irradiated up to about 1.0 Megarad, and F-II irradiated to about 0.2 Megarad, the k' values lie between 0.3–0.6, in good agreement with the requirements of Thomas. In excess of these

energy levels, however, k' for both F-I and F-II rises above 1.0. Since intrinsic viscosity is dependent on the shape of the molecule in solution and the shape in turn depends on solute:solvent interaction, k' values vary with solvent power and are inversely proportional to intrinsic viscosity. This would implicate 2 *M* KCNS as an inadequate solvent for measuring viscosity of gelatin irradiated at levels greater than 0.5 Megarad.

Nevertheless, on the basis of favorable experiments with 2 *M* KCNS by others (3) and data obtained by us, this solvent was elected and found to be quite suitable for viscosity as well as sedimentation studies. In 1% concentration, F-I and F-II gave reproducible straight-line plots of η_{sp}/c *vs.* c at 50° and at 25°. Constant values could not be obtained with either F-I or F-II when 5% solutions and films were diluted to 1% concentrations. This would appear to confirm the admonition that comparisons cannot be made on gelatins whose thermal, solution, and aging histories are not identical. Deviations invariably give misleading results because configurations of the protein molecule are not the same in sol and in gel states. For example, gelatin films prepared by hot evaporation are in a fold where hydrogen bonds are intramolecular, whereas cold-evaporated gelatin contains intermolecular hydrogen bonds and forms more stable films (18). Similar reasoning would apply to comparison of 1% and 5% gelatin solutions. In the former, solution can be effected more readily, and the molecular state is characterized by relative freedom of motion, whereas the latter requires a somewhat longer period for final dissolution of the protein, which is more compact and has less movement capability.

Therefore viscosity studies on dilute solutions, gels, and films are not susceptible to comparison even though the same protein is involved because the preparation of more concentrated forms followed by dilution, but interrupted by a short aging period, disorients the molecule. The configuration of the converted form is not the same as that in the 1% solution prepared initially. Hence, the behavior of the final solutions in viscosity studies differ appreciably. This reasoning is supported by differences in viscosity behavior of the dilute solutions, and gels and films converted to sols, following exposure to low order irradiation. The 1% solutions of F-I and F-II gave sharply definable plots of η_{sp}/c *vs.* c, readily extrapolatable to zero concentration to yield $[\eta]$. Irradiated gels and films converted to 1% solutions gave erratic results—overlapping straight-line plots and low $[\eta]$ values which could not be reproduced. It appears that irradiation doses of the order 0.4–2.5 Megarads are capable of inducing discrete physical changes in the molecular make-up of gelatin in 1% solutions. The order of change in gels and solid states is comparatively small. Because of their compactness in 5% solution and their total immobility in the solid state, impingement of small quanta of ionizing radiation on gelatin molecules in these forms fails to disrupt their makeup to a degree sufficient for useful application of viscometric measurements.

The characteristically progressive reduction in $[\eta]$ values for F-I and the shifting viscosity behavior of F-II may be attributed to changes in the size and shape of the molecules and alterations in net charge with increased application of irradiation dosage. If scission or crosslinking (or both) occur, the increase or decrease in molecular weight is a function of scission and crosslinking rates which, in turn, are proportional to radiation intensity (19). The probability of crosslinking is proportional to the integrated dose R (in Megarads) of the radiation

$$q = \alpha R$$

where α is a proportionality constant having units of mass/energy. With respect to scission

$$p = P_0 + \beta R$$

where β, the proportionality constant for scission, has the dimensions of α, and p is the overall scission probability. Shultz (20) has evaluated the ratio β/α in terms of the effect of radiation-induced branching on intrinsic viscosity in the pregelation region. By plotting $[\eta]_{\text{branched}}/[\eta]_{\text{unbranched}}$ as a function of R/R^* where R^* is the critical radiation dose, it was shown that when $\beta/\alpha = 0$ no scission occurs, $[\eta]$ increases until R approaches R^*, then drops. The drop occurs sooner as the scission gains dominance. When $\beta/\alpha > 1$, $[\eta]$ decreases at the start, although molecular weight is increasing. The latter behavior is precisely that of F-II.

It is apparent that one cannot rely exclusively on intrinsic viscosity data to interpret changes in molecular weights of irradiated proteins. A more conservative approach would be to assume that several events occur during irradiation and that the final effects on the protein represent the total of the transformations on the entire molecule which would include scission, crosslinking, endlinking, charge transfer, polymerization, and deaggregation.

The drop in intrinsic viscosity of F-II with concurrent increase in molecular weight tends to support the proposition that if the β/α ratio (20) is greater than unity, $[\eta]$ decreases initially though the molecular weight is increasing, and that full reliance cannot be placed on $[\eta]$ values to interpret changes in molecular weights of irradiated polymers. Here again one must look to the three principal reactions which may occur following free radical formation. In the lower molecular weight F-II, the lowest radiation level causes an immediate twofold increase in molecular weight. At the next level, the molecular weight becomes substantially greater and continues in the same direction. This would indicate recombination and coupling reactions between free radicals resulting in copolymerization by crosslinking and/or endlinking.

The interdependence of intrinsic viscosity, sedimentation constant, and molecular weight values for irradiated F-I and F-II has been established. Gouinlock's F-2 and F-3 fractions (7) showed the same relationship as did irradiated F-I—namely, a decrease in $[\eta]$ reflected a decrease with respect to both S^0 and M_w. The reverse pattern, nevertheless interdependent, was manifested in irradiated F-II. If it is accepted that 2 *M* KCNS is capable of maintaining gelatin in solution in a molecularly dispersed state, it will necessarily follow that changes in intrinsic viscosity can be relied upon to reflect sedimentation and molecular weight changes. Since intrinsic viscosity is related to the size of the molecule, an increase in this value in the presence

of KCNS would indicate the presence of forces other than those responsible for aggregation to account for increases in molecular weight.

In our next and final paper in this series we will show experimental data which indicate that molecular weight changes in the two fractions studied occur through a free radical mechanism.

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Antacid Properties of Calcium, Magnesium, and Aluminum Salts of Water-Insoluble Aliphatic Acids

By STUART P. ERIKSEN†, GEORGE M. IRWIN, and JOSEPH V. SWINTOSKY

The antacid properties of several calcium, magnesium, and aluminum salts of aliphatic acids were studied. A number of the salts showed potential antacid properties *in vitro*. Of this group calcium laurate also showed promising results by *in vivo* tests in dogs and humans. Along with their acid-neutralizing capacity, higher molecular weight metal salts have several interesting physical properties, for example, their insolubility in aqueous fluids above approximately pH 5, and their neutralizing properties below this pH. They tend to float on the surface of solutions and cling to the walls of a container before they react with acidic solutions. Following reaction with hydrochloric acid, salts of higher molecular weight aliphatic acids form insoluble aliphatic acids which are reputed to retard emptying of stomach fluids. A possible drawback to the higher molecular weight salts is a lower weight-to-weight antacid capacity when compared to antacids such as calcium carbonate and aluminum hydroxide.

ALKALI AND ALKALINE earth metal salts such as carbonates, bicarbonates, and glycinates have been of considerable use over the years as antacids. They possess the ability to neutralize the acid of the stomach efficiently but in some cases replace it with a considerable concentration of hydroxide ion. Recently, the authors have investigated the antacid properties of a series of metal-organic acid salts. It appeared from consideration of their physical properties that these substances might be useful antacids with interesting "secondary" properties. The *in vitro* and *in vivo* studies of these metal salts are reported in this paper.

The calcium, magnesium, and aluminum salts of long chain aliphatic acids possess several interesting properties which might counterbalance their high equivalent weight (low acid-combining power). Since they are essentially non-wetted, they tend to float on aqueous solutions, coat wet surfaces, and offer the possibility of good protective action. They react with acid only below a characteristic pH, depending on the compound. Because the acid product of the neutralization reaction is poorly dissociated and is often relatively water-insoluble, we may consider these metal salts as hydrogen ion-exchange compounds—hydrogen and metal ions being in equilibrium with the two solid forms according to the equation

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ALKALI AND ALKALINE earth metal salts such as carbonates, bicarbonates, and glycinates have been of considerable use over the years as antacids. They possess the ability to neutralize the acid of the stomach efficiently but in some cases replace it with a considerable concentration of hydroxide ion. Recently, the authors have investigated the antacid properties of a series of metal-organic acid salts. It appeared from consideration of their physical properties that these substances might be useful antacids with interesting "secondary" properties. The *in vitro* and *in vivo* studies of these metal salts are reported in this paper.

The calcium, magnesium, and aluminum salts of long chain aliphatic acids possess several interesting properties which might counterbalance their high equivalent weight (low acid-combining power). Since they are essentially non-wetted, they tend to float on aqueous solutions, coat wet surfaces, and offer the possibility of good protective action. They react with acid only below a characteristic pH, depending on the compound. Because the acid product of the neutralization reaction is poorly dissociated and is often relatively water-insoluble, we may consider these metal salts as hydrogen ion-exchange compounds—hydrogen and metal ions being in equilibrium with the two solid forms according to the equation

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TABLE I.—ANALYSIS AND SOURCES OF ANTACID MATERIALS STUDIED

Compound[a]	Manufacturer	Analysis[b] Metal, %	Acid, %	Solvent, %
A. Commercial Compounds				
1. Calcium stearate	Matheson Coleman and Bell	100.5	95.5	2.8
2. Calcium 12-hydroxy stearate	Baker Castor Oil Co.	102.5	93.5	0.8
3. Aluminum mono-12-hydroxy-stearate	" " " "	[c]	74.2	3.7
4. Aluminum di-12-hydroxystearate	" " " "	[c]	89.6	2.6
5. Aluminum tri-12-hydroxystearate	" " " "	90.8	98.2	1.7
6. Magnesium 12-hydroxystearate	" " " "	66.6	92.8	2.7
B. Synthesized Compounds				
7. Calcium pelargonate		96.0	81.8	4.5
8. Calcium caprate		94.8	89.8	3.9
9. Calcium laurate		90.5	90.6	3.7
10. Calcium myristate		85.7	86.0	2.6
11. Calcium palmitate		92.3	91.1	3.3
12. Magnesium laurate		99.3	78.6	[d]
13. Magnesium myristate		84.6	86.3	[d]
14. Aluminum laurate		92.4	88.3	[d]
15. Calcium azelate		86.5	92.0	2.0
16. Calcium sebacate		88.4	92.9	3.0

[a] Compounds 1–6 were purchased from the manufacturers and used without purification. Compounds 7–16 were synthesized by mixing aqueous solutions of the metal chlorides and the sodium aliphatic acid soaps. The precipitated products were then washed with water and air-dried. The free acids were obtained from the following manufacturers: pelargonic, Emery Ind., Inc.; myristic, Foremost Food and Chemical; all others from Matheson Coleman and Bell. Seven other compounds were studied—aluminum hydroxide, calcium hydroxide, calcium carbonate, calcium citrate, magnesium oxide, magnesium trisilicate, and magnesium stearate. These were obtained as U.S.P. grade materials and used without further purification or analysis. [b] Solvent was calculated as per cent weight loss on drying 5 hours at 110° C. Metal determinations were done by standard EDTA titration. Acid determinations were made by titration of acid-ether extraction residue. [c] Other metals than aluminum also present. [d] Solvent not determined.

$$H^+ + \text{metal salt (solid)} \rightleftarrows \text{metal ion} + \text{organic acid (solid)}$$

The pH at which some of these salts exert their buffering effect is in the optimum range (below pH 4 to 5) for effective antacid action (1). They are essentially odorless, tasteless, and innocuous; many of the aliphatic acids are natural constituents of foodstuffs (2). Finally, the low density and poor wettability of some of these salts might decrease the efficiency with which they are emptied from the stomach (through floating and adhering to the moist stomach lining), which would result in a prolongation of antacid action.

It was our intention to study the relative reaction rates of various of these metal-aliphatic acid salts in dilute hydrochloric acid as a function of the metal ion and the chain length of the aliphatic acid used in an effort to find a candidate of sufficient potential to warrant an *in vivo* test of the ideas suggested above.

EXPERIMENTAL

Materials Used.—Pertinent data concerning the analysis and source of the compounds studied are tabulated in Table I.

pH Recording Systems.—All pH measurements were made with a Photovolt line operated pH meter, model 110, altered to permit recording on a 10 mv. Brown potentiometric recorder. The span of the recorder was adjusted to match the meter scale.

Studies in Vitro.—Studies were carried out in a 100-ml. beaker stirred at about 150 r.p.m. with a magnetic stirrer. Acid was added with a screw-driven 5-ml. hypodermic syringe at a rate of 0.23 ml. of 1.75 *N* HCl per minute (equivalent to 4 ml. 0.1 *N* HCl per minute[1]). The steps in the *in vitro* testing procedure were

1. Fifty ml. of 0.1 *N* HCl were put in the beaker and pH recording begun.
2. Five Gm. of antacid was added to the stirring acid[2] as either a powder or a glycerin-wetted suspension.
3. The pH was allowed to rise to equilibrium.
4. Acid addition was begun and continued (never longer than 30 minutes) until a trend was established.
5. The curve was evaluated using the following performance factors: (*a*) delay time, (*b*) static equilibrium pH, (*c*) dynamic equilibrium pH. The definitions of these performance factors and the methods for obtaining them are illustrated in Fig. 1.

Studies in Vivo.—Beckman glass stomach electrodes (type x800-22) were modified and used to record gastric pH and the changes caused by ingested antacid in humans and dogs. A polyethylene shield (as suggested by Harrisson, *et al.* (3),) was added to protect the tip of the glass electrode. The calomel half-cell made contact with the stomach contents through a saturated KCl-soaked thread held alongside the glass electrode lead. A fine polyethylene tube[3] (containing the KCl-soaked thread together with glass-electrode lead) was pulled through a larger polyethylene tube,[3] and the end of the thread allowed to hang out of this outer tube just above where the latter was seated on the glass electrode tip. The end of the thread and tube external to the patient was held in the side arm of a standard saturated-calomel half-cell with a small

[1] It was observed that 0.1 *N* HCl and U.S.P. simulated gastric fluid (1) gave the same results, so that for simplicity we chose to use 0.1 *N* HCl.

[2] The exact amount added is not important, provided an excess is present; however, it must be in an immediately dispersable form. (See *in vivo Studies* for powder information.)

[3] Adams Intramedic tubing, PE No. 160 and No. 340.

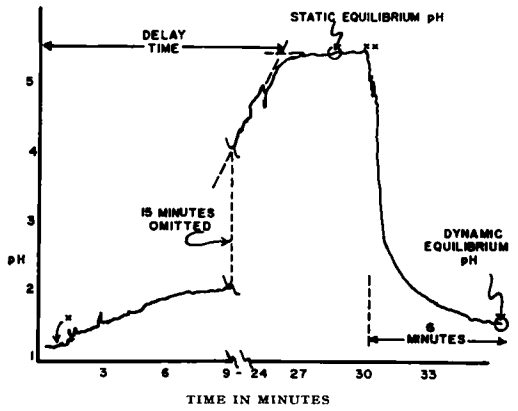

Fig. 1.—Antacid evaluation curve for magnesium stearate showing the points used to estimate the three antacid performance factors described in the text. ×, Time at which more than sufficient antacid was added to react with all of the HCl in the beaker. ××, Time at which HCl was added to stirred contents of beaker at a rate equivalent to 4 ml. 0.1 *N* HCl per minute.

cork stopper. Figure 3 is a drawing of the complete electrode system.

In vivo studies were carried out on human volunteers and anesthetized dogs. The measurement procedure used was essentially that described by Harrisson, *et al.* (3), where each subject served as his own control. Stomach pH was measured after 8 hours of fasting. (Water was given if needed.) Then the pH effects of a swallowed or intubated dose of antacid were recorded. The following suspension formula was used for all studies of powdered antacids: metal salt (5.0 Gm.), glycerin (7.0 ml.),[4] and water (30.0 ml.).

RESULTS AND DISCUSSION

The speed of response to added acid is essentially a measure of the rate of reaction with acid. If potentially longer-acting antacids are to be tested it is important that such rate of reaction studies not be done with concurrent removal of sample (simulated stomach emptying), *i.e.*, any antacid intended for prolonged action must be maintained in the stomach for the duration of its effect, either by some physical property of its own, or through special formulation.

The *in vitro* test used in this study is limited to the determination of reaction rates under two pH stresses, *i.e.*, "maximum stress" (pH 1) and "maximum rate of acid flow" (4 ml. of 0.1 *N* HCl per minue (4)). If reasonable stresses were used, it was expected that limits of response for each antacid could be observed *in vitro*, which would then correlate well with *in vivo* pH's as measured with the swallowed gastric glass electrode and would be true indications of the antacid utility of the compounds studied.

Figures 1 and 2 are tracings of pH *vs.* time records for aluminum hydroxide, calcium pelargonate, and magnesium stearate, showing the general shapes of the tracings and how the performance factors were determined. Using the performance factors shown in Fig. 1, 24 compounds were evaluated—six "simple" metal salts and hydroxides, and 18 aluminum, calcium, and magnesium salts of mono- and dicarboxylic acids of various chain lengths. The results of these *in vitro* studies are tabulated in Table II. The compounds studied show widely varying rates of reaction in acid of pH 1, the delay time for attaining static equilibrium ranging from several seconds to periods in excess of 30 minutes. The aluminum salts showed much slower rates of reaction than those of magnesium and calcium. The appearance of some rapid neutralizing effect by aluminum mono-12-hydroxystearate must be considered in the light of the presence of some other metal than aluminum (see Table I). Calcium and magnesium salts show much higher and more useful rates of reaction in acid, although the stearates in both cases seem to react slowly. All the so-called "simple" substances (hydroxide, carbonate, citrate, and trisilicate) show rapid rates of reaction. As one might expect, increase in the acid chain length slows reaction rates, though not always markedly. Dicarboxylic salts up to ten carbons show essentially the same reaction rate as the mono-basic acids.

Delay times are affected to some degree by factors such as rate of stirring, particle size, water solubility of the antacid, and degree of wetting. Although particle size may have been somewhat variable among these compounds, an effort was made to keep the rate of stirring constant and to wet the

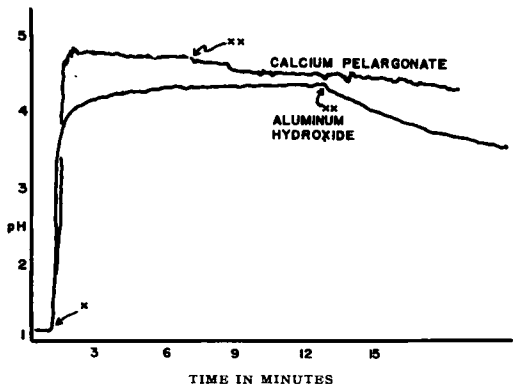

Fig. 2.—Antacid evaluation curves for aluminum hydroxide and calcium perlargonate. ×, Point of antacid addition. ××, Point at which acid input was started.

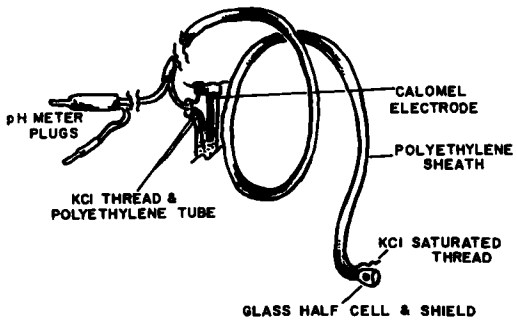

Fig. 3.—Diagram of the modified *in vivo* gastric electrode.

[4] Twenty-one ml. of glycerin was required for calcium stearate.

TABLE II.—*In Vitro* PERFORMANCE FACTORS FOR VARIOUS ANTACID SUBSTANCES

Antacid	Delay Time, Min.	Static Equilibrium, pH	Dynamic Equilibrium, pH	Dose in Gm. Calcd., 100 Min. Effect[a] 4 ml./min.	1 ml./min.
Aluminum hydroxide	<1	4.5	3.8	1.2	0.4
Calcium hydroxide	<1	12.6	12.6	1.7	0.6
Calcium carbonate	<1	5.7	5.3	2.3	0.8
Calcium citrate	<1	3.5	3.3	4.3	1.4
Magnesium oxide	<1	10.1	9.9	0.9	0.3
Magnesium trisilicate	<1	6.4	4.9	7.9	2.6
Calcium pelargonate	<1	4.8	4.6	8.0	2.7
Calcium caprate	<1	4.8	4.6	8.6	2.9
Calcium laurate	<1	4.8	4.5	9.9	3.3
Calcium myristate	1.0	4.8	4.5	11.1	3.7
Calcium palmitate	1.2	4.8	4.4	12.4	4.1
Calcium stearate	5.7	4.7	4.4	13.6	4.5
Calcium 12-hydroxystearate	2.3	5.0	4.5	14.3	4.8
Aluminum laurate	>30	...	...	9.4	3.1
Aluminum myristate	>30	...	...	10.6	3.5
Aluminum mono-12-hydroxystearate	26	3.0	1.8	5.4	1.8
Aluminum di-12-hydroxystearate	>30	...	...	9.6	3.2
Aluminum tri-12-hydroxystearate	>30	...	...	13.9	4.6
Magnesium laurate	<1	5.6	5.4	9.5	3.2
Magnesium myristate	<1	5.7	5.5	10.8	3.6
Magnesium stearate	26	5.5	1.4	13.3	4.4
Magnesium 12-hydroxystearate	4	6.1	2.5	14.0	4.7
Calcium azelate	<1	4.6	4.4	5.1	1.7
Calcium sebacate	<1	5.0	4.8	5.4	1.8

[a] Calculated on the basis of 50 ml. 0.1 *N* HCl residual acid plus 1 or 4 ml. of 0.1 *N* HCl secretion per minute for 100 minutes.

powders uniformly before determining the pH *vs.* time records.

The static equilibrium pH's reflect the pKa of the acid and the properties of the metal ion produced by neutralization. For aluminum salts the static pH's are about 3, for calcium 4.8, and for magnesium 5.5. The dynamic equilibrium pH's follow the delay times as expected; *i.e.*, where there are long delay times dynamic pH values are also low, which indicates slow response of the compound to added acid. The static pH values indicate the maximum pH's that the antacid is able to create in the stomach, while the dynamic values give an indication of the ability of an antacid to respond to stomach secretions—essentially its ability to maintain pH under high rates of acid secretion. While varying with stirring, wetting, and particle size, the dynamic equilibrium pH's are also functions of the rate of acid addition chosen. The maximum rate currently suggested (4) was selected to set a lower limit on antacid capability. The dynamic equilibrium value desired in these studies was that pH at which the rate of acid neutralization equals the rate of acid addition under a standard set of conditions. For practical reasons (sticking, clumping, etc.) this point is difficult to observe, and the pH after 6 minutes of acid addition was selected as the dynamic equilibrium value. This is recorded in Table II to permit ranking of compounds.

The final two columns of Table II are included to give an idea of the relative dose sizes of these compounds required to maintain pH at a value greater than 2 for 100 minutes under two extremes of acid production or addition (1 and 4 ml./min.).

Current opinion (1) suggests that an effective antacid should be capable of maintaining the stomach pH between 2–5. One might then conclude that compounds yielding static equilibrium and dynamic equilibrium pH's outside this range would be inferior antacids; calcium hydroxide and carbonate, magnesium oxide, tri-silicate, laurate, and 12-hydroxystearate have static pH's that fall outside this range by our test procedure.

From the table one would select calcium pelargonate, azelate, or sebacate as having optimum characteristics, *i.e.*, relatively low dose, fast onset, rapid response, and low static pH (to prevent the possibility of "acid rebound").[5] In selecting the acid moiety to be used in conjunction with the metal, an acid of low toxicity and volatility and of moderately short chain length is desirable.[6] Minimal volatility is desirable to avoid taste and odor from the free acid formed during the *in vivo* neutralization reaction. Aliphatic acids of less than 12 carbon atoms are liquid at body temperature and have disagreeable odors. The 12-carbon lauric acid is solid at body temperature and is virtually free of odor, and its calcium salt is an antacid with properties making it a potentially useful antacid. Thus, calcium laurate was selected for *in vivo* studies with calcium carbonate as the control.

In order to circumvent any membrane potential error, which the authors and others (6, 7) have observed to amount to about 30 mv. or $^1/_2$ pH unit, *in vivo* studies were not done with the calomel cell on the arm (3) or in the mouth. Direct calomel contact with the gastric contents, even by such elementary means as we have described, permits true pH values to be obtained inside or outside the body.

A summary of the data obtained from *in vivo* studies on three dogs and three humans is shown in Table III. Two conclusions may be drawn from these *in vivo* tests: (*a*) calcium laurate is an effective antacid, and (*b*), though its duration appears to be

[5] We could not demonstrate "rebound" in any of our studies on dogs and humans.

[6] Though the toxicity of azelaic and sebacic acids is not well understood, they have been reported to be *nephrotoxic* in animals (5) and therefore were not considered for the *in vivo* test.

TABLE III.—SUMMARY OF ANTACID DATA OBTAINED FROM SEVERAL ANTACIDS BY THE *in Vivo* ELECTRODE RECORDING TECHNIQUE, USING DOGS AND HUMANS

	Calcium Laurate			Control			
	Dose, Gm.	Max. pH	Duration,[a] min.		Dose, Gm.	Max. pH	Duration,[a] min.
Dog 1	0.5	7.0	70	Na_2CO_3	0.02	8.2	60
Dog 2	2.5	5.0	[b]	$CaCO_3$	0.19	6.0	65
Dog 3	2.5	4.8	>60[c]	$Mg(OH)_2$	0.10	7.7	30
Patient 1	3	4.6	65	...	...	...	...
Patient 2	2.5	4.8	80	...	...	...	...
Patient 3	5.0	5.1	100–130	$CaCO_3$	1.14	6.2	80

[a] Minutes after ingestion until gastric pH reached two. [b] Defective operation of pH meter. [c] Recording was stopped after 60 minutes.

longer in a human than an equivalent amount of calcium carbonate control, the difference is probably not significant. One might extrapolate this to mean that the principle of "floating" or adhering through poor wetting does not enhance stomach retention time markedly, although considerable more controlled *in vivo* work would be required to prove this conclusively.

SUMMARY

1. The following compounds were evaluated *in vitro* for antacid activity: aluminum hydroxide, aluminum laurate, aluminum myristate, aluminum mono-12-hydroxystearate, aluminum di-12-hydroxystearate, calcium hydroxide, calcium pelargonate, calcium citrate, calcium carbonate, calcium caprate, calcium laurate, calcium myristate, calcium palmitate, calcium stearate, calcium 12-hydroxystearate, calcium azelate, calcium sebacate, magnesium oxide, magnesium trisilicate, magnesium laurate, magnesium myristate, magnesium stearate, and magnesium 12-hydroxystearate.

2. In the *in vitro* studies, pH *vs.* time recordings were made under conditions which allowed evaluation of performance. Performance factors were designated (a) *delay time*, (b) *static equilibrium pH*, and (c) *dynamic equilibrium pH*. These factors indicated under the experimental conditions employed (*a*) the time required for an excess of the antacid to raise the pH of a 0.1 *N* HCl solution to an equilibrium value, (*b*) the equilibrium pH value when no further HCl was added to the system, and (*c*) the approximate equilibrium pH resulting (under conditions of excess antacid) when acid was added continuously at a rate equivalent to 4 ml. 0.1 *N* HCl per minute.

3. Most of the salts of carboxylic acids used in these studies have not been reported previously to be antacid substances, yet they all appear to possess this property. Their antacid properties result from their ability to react with HCl and form the free carboxylic acids. These free acids are poorly dissociated and many are insoluble in aqueous fluids. Thus their formation results in removal of hydrogen ions from solution.

4. The pH's below which these salts exert antacid action are influenced in part by the pKa of the carboxylic acid and the nature of the metal cation. For the aluminum, calcium, and magnesium salts studied, effective antacid actions were exerted at pH's near 4, 4.8, and 5.5, respectively.

5. Rate of acid neutralization *in vitro* appears to be influenced by factors such as rate of stirring, particle size of the antacid, its water solubility, and its wettability. Rates of acid neutralization cannot be predicted beforehand; however, in general, for salts derived from a given metal, the lower equivalent weight salts reacted more rapidly than those of higher equivalent weight. For example, calcium pelargonate or calcium azelate neutralized HCl much more rapidly than did calcium stearate.

6. Limited studies were performed in anesthetized dogs and in humans using a stomach electrode. Calcium laurate (a typical salt of a carboxylic acid) was shown to possess antacid properties *in vivo*.

7. One can infer from these studies that many salts of carboxylic acids possess antacid properties; however, none of the salts studied appeared to offer general advantage over some commonly used antacids. The principal drawback of the salts derived from the higher molecular weight carboxylic acids is the relatively high doses that would be required in practical therapy.

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Effect of Various Compounds on the Rate of Thiamine Hydrolysis

By JOHN J. WINDHEUSER and TAKERU HIGUCHI

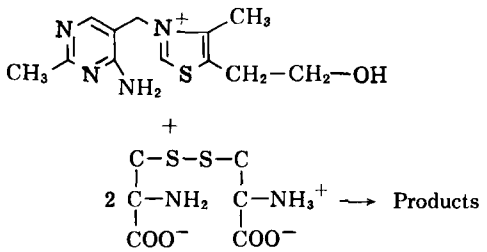
The catalytic effects of a variety of compounds on the rate of thiamine hydrolysis have been investigated. Although some catalysis was observed, thiamine degradation does not appear to be particularly sensitive to the presence of a number of nucleophilic agents other than sulfite and thiosulfate.

In our previous publication (1) dealing with the kinetics of thiamine hydrolysis, reference was made to the reports by others (2) indicating that certain compounds exhibited remarkable positive or negative catalytic effects on the rate of hydrolysis of this vitamin. Although investigation of some of these compounds under our experimental conditions failed to substantiate the reported dramatic effects, the possible theoretical and technical importance of finding catalytic agents has motivated further investigation in this area. The present communication is concerned with the effects of several amino acids and other compounds on the rate of thiamine cleavage at 96.4°.

RESULTS AND DISCUSSION

Amino Acids.—It has been reported in the literature (3) that the destruction of thiamine by shrimp thiaminase was retarded by the addition of cystine at pH 6.9. In order to determine whether the reported effect might be due to an interaction of cystine with thiamine resulting in stabilization or due to the amino acid lowering the catalyst activity, thiamine was subjected to hydrolytic conditions in the presence of various concentrations of cystine. Experiments were conducted at 96.4° under anaerobic conditions, at constant pH, ionic strength, and buffer concentration.

The results of studies at pH 6.3 are shown in Fig. 1. The increase of the reaction rate as a function of the cystine concentration indicates that rather than stabilizing thiamine, cystine actually acts to catalyze the degradation. The nonlinearity of the increase in reaction rate at rising amino acid titer suggests a higher order dependency of the reaction on cystine. Based on the observed pH dependence, the solid line on the graph was calculated on the assumption that the reaction could be represented as



From such a formulation it can be readily shown that

$$k_{obs} = k_o + k_{cy}\left[cy_t\left(\frac{K_a}{H^+ + K_a}\right)\right]^2$$

where k_{obs} is the observed pseudo first order rate constant, k_o is the first order rate constant at the experimental pH and buffer concentration, k_{cy} is the specific catalytic constant for cystine, cy_t represents the total cystine added to the system, and K_a denotes the acidic dissociation constant of the first amino group. The good agreement of the experimental points with the theoretical curve tends to support the proposed reaction dependency.

Figure 2 depicts the results obtained from the same reaction run at pH 6.69. The solid line again represents a theoretical curve calculated with the above equation utilizing the catalytic constant estimated from the reaction at pH 6.3.

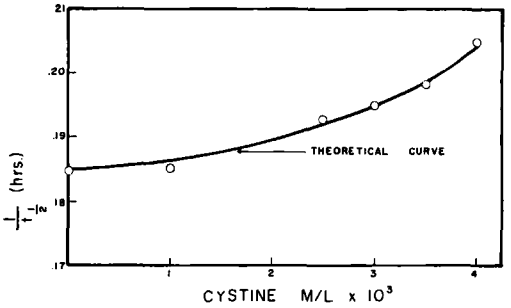


Fig. 1.—The effect of increasing cystine concentration on the rate of thiamine hydrolysis at pH 6.3, using 0.1 *M* phosphate buffer at an ionic strength of 0.5.

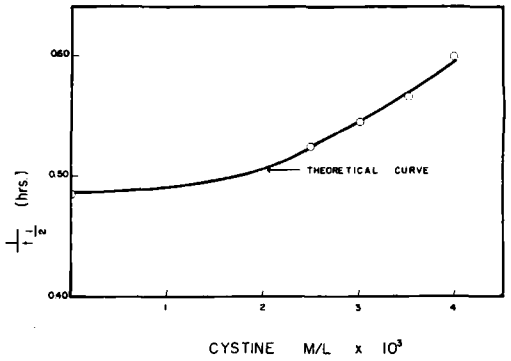


Fig. 2.—The effect of increasing cystine concentration on the rate of thiamine hydrolysis at pH 6.69 using 0.1 *M* phosphate buffer at an ionic strength of 0.5.

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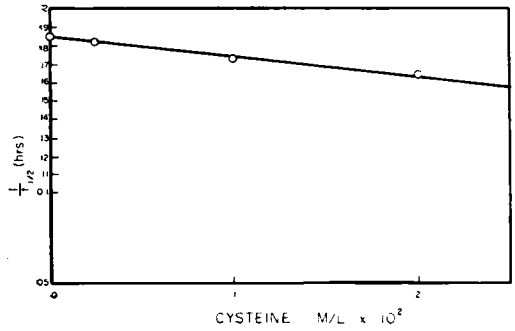


Fig. 3.—The effect of cysteine on the rate of hydrolysis at pH 6.3 using 0.1 *M* phosphate buffer at an ionic strength of 0.5.

TABLE I.—THE EFFECT OF VARIOUS AMINO ACIDS ON THE RATE OF THIAMINE HYDROLYSIS AT 96.4° IN THE PRESENCE OF 0.1 *M* PHOSPHATE BUFFER AT 0.5 IONIC STRENGTH

Compound	pH	Apparent Catalytic Constant, LM^{-1} $Hr.^{-1}$
Cystine	6.30	2.08×10^{6a}
Cystine	6.69	2.08×10^{6a}
Cysteine	6.30	-8.5×10^{-1b}
Tyrosine	6.30	...

[a] Calculated on the basis of Eq. 1 and using $pK_3 = 8.0$ for cystine. [b] Based on total cysteine concentration.

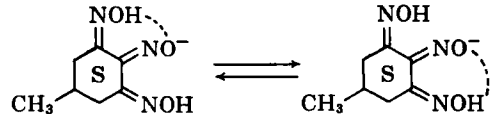
From the results of these experiments, which indicate that cystine *per se* catalyzes the decomposition of thiamine, it would appear probable that the retardation of the degradation of thiamine by thiaminase in the presence of cystine does not involve interaction of thiamine and cystine but rather an interaction of the amino acid with thiaminase.

The possible interconversion of cystine to cysteine under redox conditions led to the investigation of the behavior of cysteine. The results, as shown in Fig. 3, indicate that cysteine exhibits a definite inhibitory effect on the rate of cleavage. The apparent first-order dependence on cysteine and retardation of the rate of degradation, as compared to the results with cystine, would tend to suggest that the free sulfhydryl group is vital to the stabilizing mechanism. This is further substantiated when cysteine is compared to alanine which in our previous work (1) has been found to increase the rate of degradation. These findings would be in agreement with those of Watanabe (4) who reported that a number of sulfhydryl-containing compounds were found to prevent the formation of turbidity in thiamine solutions heated at 100° for three hours.

Although the structure of thiaminase has as yet not been elucidated, it has been shown that tyrosine is one of the amino acid components (5). Therefore experiments were carried out to determine if tyrosine might be one of the components of the active sites of this enzyme. The studies were conducted under the same conditions noted previously, and it was found that this compound exhibited no catalytic activity.

The results observed with the amino acids tested are summarized in Table I.

Carbonyl Derivatives.—Since it has been shown that thiamine hydrolysis is subject to general base catalysis, a number of ketones, ketoximes, and a triketo triazine were tested as each of these compounds is capable of acting as a nucleophilic catalyst, as has been shown by Russo (6) in his study of the hydrolysis of phosphate esters. Table II lists the compounds of this group that were tested and the catalytic constants observed. Figure 4 represents the kinetic dependency of the thiamine cleavage on the concentrations of cyclohexane oxime and 5-methyl-1,2,3-cyclohexanetrione-1,3-dioxime. In each case the reaction appears to be first order with respect to the catalytic agents. The higher catalytic constant of the dioxime may be due to a bifunctional attack of the weak nucleophilic and electrophilic centers in the molecule. The lack of activity of trioximes might be rationalized by the formation of intramolecularly bonded salts at this pH as follows



This supposition appears to be supported by the fact that the salicylate ion as well as the 2-hydroxy-1-naphthoate ion, both of which can form similar internal associations, exhibited no catalytic action.

TABLE II.—EFFECT OF VARIOUS COMPOUNDS ON THE RATE OF THIAMINE HYDROLYSIS AT PH 6.30 AND 96.4° IN THE PRESENCE OF 0.1 *M* PHOSPHATE BUFFER AND 0.5 IONIC STRENGTH

Compound	Apparent Catalytic Constant, LM^{-1} $Hr.^{-1}$	Max. Concn. *M*/L.
Cyclohexanone	No activity	1×10^{-2}
Cyclohexanone oxime	1.18	5×10^{-2}
1,2,3-Cyclohexanetrione trioxime	No activity	1×10^{-2}
5-Methyl-1,2,3-cyclohexanetrione 1,3 dioxime	2.46	3.5×10^{-2}
5-Methyl-1,2,3-cyclohexanetrione trioxime	No activity	1×10^{-2}
Trimethyl isocyanuric ester	No activity	1×10^{-2}

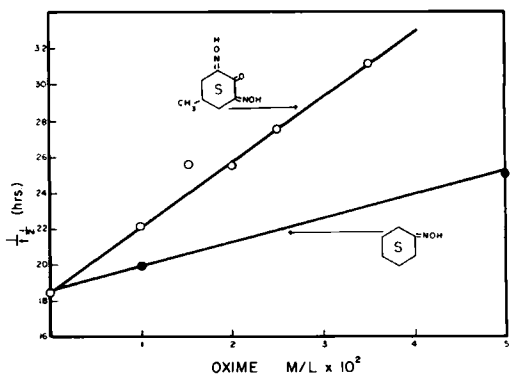


Fig. 4.—The effect of cyclohexanoxime and 5-methyl-1,2,3-cyclohexanetrione 1,3-dioxime on the rate of thiamine hydrolysis at pH 6.3 using 0.1 *M* phosphate buffer at an ionic strength of 0.5.

Other Compounds.—In addition to the amino acids and the agents tabulated in Table II, catechol, aniline, and 2-hydroxy-1-naphthoic acid were evaluated. None of these compounds altered the rate of the reaction.

EXPERIMENTAL

Reagents.—All chemicals used in the preparation of the buffers and the kinetic solutions were analytical reagent grade with the exception of the catalytic agents tested. These compounds were all purified by recrystallization from suitable solvents prior to use.

Analytical Procedure.—Residual thiamine was determined by the U.S.P. XVI (7) thiochrome method. All substances added to the system were checked for interference with the assay method.

Kinetic Procedure.—The procedure for the kinetic runs was identical with that previously described (1).

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Kinetics of Air Oxidation of Sulfurous Acid Salts

By LOUIS C. SCHROETER

Sulfurous acid salts in common with many pharmaceutical antioxidants and oxygen-sensitive drugs undergo oxidation by a radical process. This has important implications in the testing and evaluation of oxidative tendencies of formulations since radical processes are inordinately sensitive to slight amounts (10^{-6} *M*) of compounds acting as radical inhibitors or initiators. Measurable inhibition of the rate of sulfite oxidation occurred in the presence of 10^{-6} *M* DMA or DMF; the inhibition is described by an empirical equation. Effect of hydrogen ion concentration on the oxidation of sulfurous acid salts was studied in detail and the results described in terms of a theoretically derived equation. Experimental data are presented in corroboration of the Abel theoretical equations and radical mechanism for sulfite oxidation.

SULFUROUS ACID salts in aqueous solution undergo oxidation in the presence of molecular oxygen by a radical process (1). Radical initiation very likely occurs through interaction of oxygen with an anion to produce free radicals

$$(I)\ O_2 + OH^- \rightleftharpoons OH + O_2^-(O_3H^-)$$

which in turn react with hydrogen sulfite species to produce a highly reactive radical.

$$(II)\ HSO_3^- + OH \rightarrow HSO_3 + OH^-$$

The product of the oxidation, sulfate ion (SO_4^{2-}), may be formed by the interaction of HSO_3 and OH radicals

$$(III)\ OH + HSO_3 \rightarrow SO_4^{2-} + 2H^+$$

Other mechanistic interpretations consistent with the concept of a radical process and conforming to the energetics of the system may adequately explain the experimental data. Consideration of plausible radical mechanism is aided by reviewing treatment accorded to other radical systems (2) and especially to free radical reactions occurring in solution (3).

Abel (4, 5) has proposed the following scheme for generalized anionic auto-oxidation

$$O_2 + A^- \rightarrow AOO^-$$
$$AOO^- + X \rightarrow 2O^- + X^+ + A$$
$$A + X \rightarrow X^+ + A^-$$

in which the hydroxyl ion appears to be the preferred electron donor, A^-. In this scheme X would represent hydrogen sulfite species, HSO_3^-.

Radical processes are extraordinarily sensitive to small amounts of compounds which may act to inhibit or to catalyze the reaction. Addition of sulfurous acid salts to pharmaceutical formulations generally results in rather complex systems from the standpoint of free radical chemistry. Drug molecules or other additives in the formulation may act as initiators, inhibitors, or catalysts of the oxidative process. Systems which decrease the efficiency of the antioxidant ultimately result in poor stability of the drug since failure of the antioxidant is generally followed by oxidative attack on the drug.

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Other Compounds.—In addition to the amino acids and the agents tabulated in Table II, catechol, aniline, and 2-hydroxy-1-naphthoic acid were evaluated. None of these compounds altered the rate of the reaction.

EXPERIMENTAL

Reagents.—All chemicals used in the preparation of the buffers and the kinetic solutions were analytical reagent grade with the exception of the catalytic agents tested. These compounds were all purified by recrystallization from suitable solvents prior to use.

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Kinetics of Air Oxidation of Sulfurous Acid Salts

By LOUIS C. SCHROETER

Sulfurous acid salts in common with many pharmaceutical antioxidants and oxygen-sensitive drugs undergo oxidation by a radical process. This has important implications in the testing and evaluation of oxidative tendencies of formulations since radical processes are inordinately sensitive to slight amounts (10^{-6} *M*) of compounds acting as radical inhibitors or initiators. Measurable inhibition of the rate of sulfite oxidation occurred in the presence of 10^{-6} *M* DMA or DMF; the inhibition is described by an empirical equation. Effect of hydrogen ion concentration on the oxidation of sulfurous acid salts was studied in detail and the results described in terms of a theoretically derived equation. Experimental data are presented in corroboration of the Abel theoretical equations and radical mechanism for sulfite oxidation.

SULFUROUS ACID salts in aqueous solution undergo oxidation in the presence of molecular oxygen by a radical process (1). Radical initiation very likely occurs through interaction of oxygen with an anion to produce free radicals

$$(I)\ O_2 + OH^- \rightleftharpoons OH + O_2^-(O_3H^-)$$

which in turn react with hydrogen sulfite species to produce a highly reactive radical.

$$(II)\ HSO_3^- + OH \rightarrow HSO_3 + OH^-$$

The product of the oxidation, sulfate ion (SO_4^{2-}), may be formed by the interaction of HSO_3 and OH radicals

$$(III)\ OH + HSO_3 \rightarrow SO_4^{2-} + 2H^+$$

Other mechanistic interpretations consistent with the concept of a radical process and conforming to the energetics of the system may adequately explain the experimental data. Consideration of plausible radical mechanism is aided by reviewing treatment accorded to other radical systems (2) and especially to free radical reactions occurring in solution (3).

Abel (4, 5) has proposed the following scheme for generalized anionic auto-oxidation

$$O_2 + A^- \rightarrow AOO^-$$
$$AOO^- + X \rightarrow 2O^- + X^+ + A$$
$$A + X \rightarrow X^+ + A^-$$

in which the hydroxyl ion appears to be the preferred electron donor, A^-. In this scheme X would represent hydrogen sulfite species, HSO_3^-.

Radical processes are extraordinarily sensitive to small amounts of compounds which may act to inhibit or to catalyze the reaction. Addition of sulfurous acid salts to pharmaceutical formulations generally results in rather complex systems from the standpoint of free radical chemistry. Drug molecules or other additives in the formulation may act as initiators, inhibitors, or catalysts of the oxidative process. Systems which decrease the efficiency of the antioxidant ultimately result in poor stability of the drug since failure of the antioxidant is generally followed by oxidative attack on the drug.

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Antioxidants are added to pharmaceutical formulations as redox systems possessing higher oxidative potentials than the drug which they are designed to protect or as chain inhibitors of radical induced decomposition (6). Selection of the antioxidant is often based on sound theoretical grounds, such as the difference in redox potential between the drug and the compound; however, electrometric measurements only rarely serve to predict actual efficiency of antioxidants in complex pharmaceutical systems. Kinetics of the oxidation process and radical inhibitory or initiating action of the drug and/or the antioxidant cannot be deduced from redox potentials. Obviously, the most reliable information is obtained under field conditions (7). Laboratory evaluation of antioxidants in pharmaceutical formulations by exposing the system to oxygen under controlled conditions is a more realistic and dependable method. Manometric techniques have found considerable favor among investigators studying oxidations and auto-oxidations. Jorissen (8) describes typical manometric procedures in his interesting text on induced oxidation. Manometric techniques suffer from three main disadvantages: (*a*) in complex systems containing several oxidizable molecules or species it is not possible to assign oxygen uptake to a specific compound; (*b*) it is operationally difficult to assay the solution by an independent method while the experiment is underway; and (*c*) it is difficult to provide adequate stirring of the solution in contact with oxygen.

Many of the reported studies which have used manometric techniques are of questionable value inasmuch as critical evaluation of experimental techniques indicates that the diffusion rate of oxygen was the rate-determining step. If oxidation is sufficiently rapid, and if the stirring of the solution is slow, the rate at which oxygen is dissolved into the solvent determines how rapidly the system is oxidized.

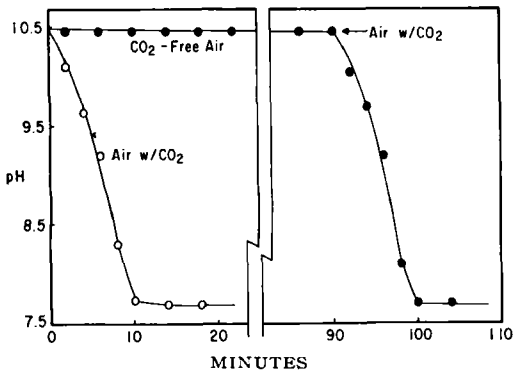

Fig. 1.—Effectiveness of gas scrubbers in removing CO_2 from air supply. Standard reactor containing 300 ml. water with 0.1 meq. NaOH stirred at 1000 r.p.m. at 25° with air flow rate of 50 cm.^{3} sec.$^{-1}$.

TABLE I.—EFFECT OF AIR FLOW RATE ON OXIDATION OF 0.02 *M* SODIUM SULFITE AT 25° IN STANDARD REACTOR

Air Flow Rate[a]	Initial pH	Reaction, %	Final pH	Specific First-Order Constant
25 cm.^{3} sec.$^{-1}$	9.4	90	7.4	2.2×10^{-3} sec.$^{-1}$
38 cm.^{3} sec.$^{-1}$	9.4	88	7.3	2.8×10^{-3} sec.$^{-1}$
50 cm.^{3} sec.$^{-1}$	9.3	94	7.4	2.9×10^{-3} sec.$^{-1}$
62 cm.^{3} sec.$^{-1}$	9.4	93	7.3	3.0×10^{-3} sec.$^{-1}$

[a] CO_2-free air.

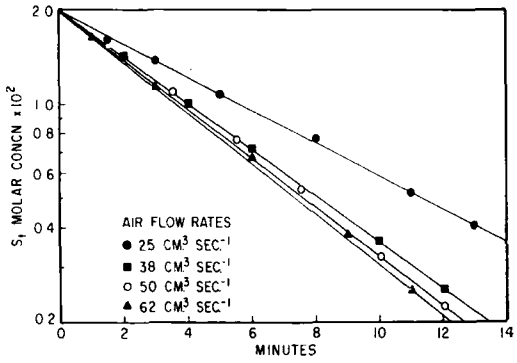

Fig. 2.—Effect of air flow rates on oxidation rate of 0.02 *M* sodium sulfite at 25° in standard reactor. Solutions stirred at 1000 r.p.m. CO_2-free air saturated with water at 25°.

The comparative value of various antioxidants in protecting drug formulations is best accomplished by subjecting the system to standard oxidative conditions and periodically assaying the formulation for both drug and antioxidant. This procedure requires a maximum of effort but yields the most useful information for rational formulating. Complexity of radical oxidative processes and their sensitivity to small amounts of material place stringent limitations on the validity of comparisons among different pharmaceutical systems.

This study was designed to evaluate the operation of a standard air oxidation reactor with aqueous sulfurous acid salts and to make critical comparisons with theoretically derived kinetic data (9).

EXPERIMENTAL

Apparatus and Material.—Air oxidation of sodium sulfite solutions was carried out in a Pyrex vessel with a volume of approximately 600 ml. The vessel was provided with a gas dispersion tube, sampling port, short condenser, and a glass stirrer in a ground-glass bearing. Stirring speeds could be varied up to 1000 r.p.m. The reaction vessel was immersed in a thermostat maintained at 25 ± 0.01°.

Air supplied to the reactor was passed through a filter and molecular sieve to remove suspended solid and liquid particles. The air stream was then conducted through three gas scrubbing towers in series maintained in a thermostat at the same tem-

TABLE II.—SUMMARY OF FIRST-ORDER SPECIFIC RATE CONSTANTS[a]

Sulfite Sample[b]	Initial Concn., M	No. Runs	Av. % Completion	k (sec.$^{-1}$)
A	0.02	3	80	2.4×10^{-3}
B	0.02	2	75	2.8×10^{-3}
C	0.02	2	80	3.7×10^{-3}
D	0.02	2	90	2.6×10^{-3}
E	0.02	2	70	2.8×10^{-3}
			Av.	2.86×10^{-3} sec.$^{-1}$

[a] Standard 500-ml. Pyrex reactor stirred at 1000 r.p.m. containing 300 ml. solution. Air (CO_2-free) saturated with water at 25° bubbled through solution at rate of 50 cm.^{3} sec.$^{-1}$. [b] Sample *A*—Fisher certified reagent sodium sulfite; *B* sample prepared by 2 × recrystallization of *A*; *C* sample—sodium sulfite N.F., Matheson Coleman and Bell; *D* sample prepared by 1 × recrystallization of *C*; *E* sample prepared by 1 × recrystallization of *D*.

perature as the reactor. Air flow rate was measured with a calibrated wet gas flow meter[1] and maintained within 5 cm.^{3} sec.$^{-1}$ of the desired flow rate. Tubing was Pyrex glass with short pieces of Tygon used for connections.

Water was distilled twice from a Pyrex apparatus and stored not longer than 12 hours under nitrogen atmosphere. Prior to use, this water was once again distilled and used within 4 hours for making solutions, filling gas scrubbers, and rinsing equipment. Sodium sulfite was recrystallized from distilled water and dried at 100° under vacuum. N,N-dimethylformamide and N,N-dimethylacetamide were spectroquality reagents (Matheson Coleman, and Bell).

Carbon Dioxide Removal from Air Supply.—Air was passed through three thermostated scrubbing towers containing water; flow rate was maintained at 50 ± 5 cm.^{3} sec.$^{-1}$ This air was conducted through the standard reactor which contained 0.1 meq. sodium hydroxide in 300 ml. triple distilled water. The reactor was maintained at 25 ± 0.01° and stirred at 1000 r.p.m. Samples were periodically removed for pH measurement. The procedure was repeated with another 300-ml. volume of solution containing 0.1 meq. hydroxide ion through which air scrubbed free from carbon dioxide by passage through (*a*) sodium hydroxide solution, (*b*) sulfuric acid solution, and (*c*) triple distilled water was conducted. After 90 minutes with no significant change in pH of the basic solution, air supply to the reactor was conducted through water scrubbers.

Effect of Air Flow Rate.—Three-hundred ml. of 0.02 *M* sodium sulfite contained in the standard reactor was stirred at 1000 r.p.m. at 25 ± 0.01°. Air (CO_2 free) was introduced through a medium glass frit beneath the surface of the stirred fluid at varying flow rates. Samples were removed periodically through the sample port with a syringe. Total sulfite concentration was determined iodometrically. The pH was determined using a Beckman GS pH meter.

Sulfite as a Function of Time.—The standard reactor was used for all studies. Three-hundred ml. of sulfite solution was stirred at 1000 r.p.m. at 25 ± 0.01° while water-saturated, carbon dioxide-free air was introduced at 50 ± 5 cm.^{3} sec.$^{-1}$ beneath the surface of the fluid. Total sulfite concentration of samples was determined iodometrically. The pH

[1] Flowrator, Fischer-Porter Co., Hatboro, Pa.

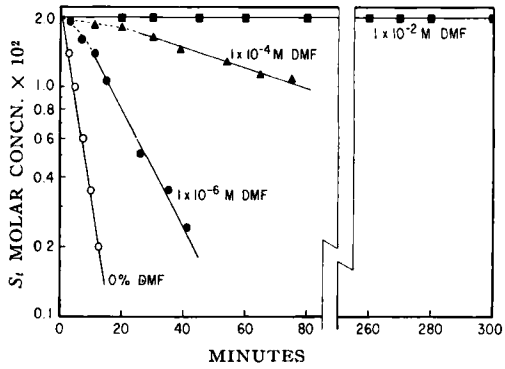

Fig. 3.—Inhibitory action of N,N-dimethylformamide (DMF) on sulfite oxidation (0.02 *M*) in standard reactor stirred at 1000 r.p.m. at 25°. Air (CO_2-free) flow rate: 50 cm.^{3} sec.$^{-1}$

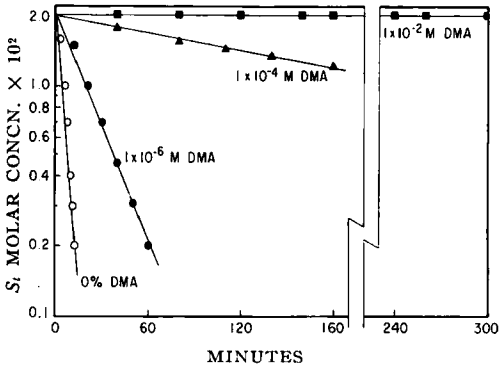

Fig. 4.—Inhibitory action of N,N-dimethylacetamide (DMA) on sulfite oxidation (0.02 *M*) in standard reactor stirred at 1000 r.p.m. at 25°. Air (CO_2-free) flow rate: 50 cm.^{3} sec.$^{-1}$

of samples removed from the reactor was determined with a Beckman GS pH meter.

RESULTS AND DISCUSSION

Removal of carbon dioxide as well as acidic or basic contaminants from air supplied to the reactor was especially important inasmuch as the aqueous sulfurous acid system is known to be sensitive to a wide variety of inhibitors and catalysts. Air contains about 0.03% by volume of carbon dioxide; thus, air flow rates around 3 L./min. result in the introduction of about 1 ml. of carbon dioxide per minute into the reactor. Effectiveness of the gas

TABLE III.—INHIBITORY EFFECT OF DMA AND DMF ON AIR OXIDATION OF SULFITE (0.02 *M*) IN STANDARD REACTOR[a] AT 25°

Inhibitor, M concn..	Completion, %	k_2 (sec.$^{-1}$)	k_2/k_1	$\left(\frac{A}{B+m}\right)^c$
0	90	2.88×10^{-3}	1.00	1.00
1×10^{-6} DMF	88	1.05×10^{-3}	0.365	0.45
1×10^{-4} DMF	72	1.78×10^{-4}	0.062	0.05
1×10^{-2} DMF	0[b]	...	...	...
1×10^{-6} DMA	85	6.4×10^{-4}	0.222	0.182
1×10^{-4} DMA	75	5.3×10^{-5}	0.018	0.018
1×10^{-2} DMA	0[b]	...	...	...

[a] Solution stirred at 1000 r.p.m. Air (CO_2-free) flow rate: 50 cm.^{3} sec.$^{-1}$. [b] No reaction detectable after 380 minutes. [c] m = molar concentration of inhibitor; A and B constants for each inhibitor: (DMF): $A = 0.5 \times 10^{-6}$, $B = 1 \times 10^{-6}$; (DMA): $A = 0.25 \times 10^{-6}$, $B = 1 \times 10^{-6}$.

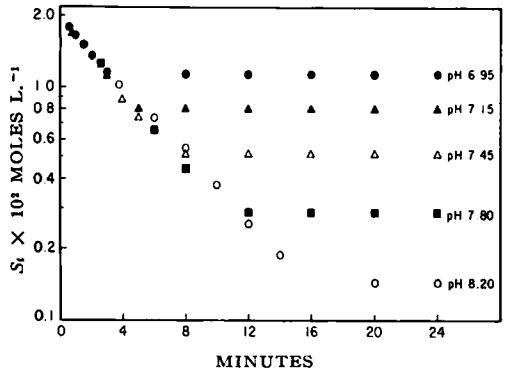

Fig. 5.—Effect of initial pH on oxidation rate of 0.02 *M* sodium sulfite at 25°. Standard reactor stirred at 1000 r.p.m. with CO_2-free air (50 cm.^{3} sec.$^{-1}$).

scrubbers in removing carbon dioxide from the air supply is shown in Fig. 1. Air introduced at a rate of 3 L./min. into a weakly basic solution contained in the reactor caused a rapid increase in hydrogen ion concentration of the solution; however, passing air through gas scrubbers effectively removed carbon dioxide: the pH of the solution changed little even after 270 L. of air had passed through it.

Preliminary tests showed that diffusion of oxygen was not a controlling factor with stirring rates greater than about 800 r.p.m. when the air flow rate was maintained at approximately 50 cm.^{3}/sec. Effect of air flow rate when the rate of stirring was held constant at 1000 r.p.m. is shown in Table I and Fig. 2. Flow rates greater than 38 cm.^{3} sec.$^{-1}$ appear to cause no significant variation in the rate of oxidation. It was therefore concluded that diffusion of oxygen was not a controlling factor in experiments in which the air flow rate was maintained at 50 ± 5 cm.^{3} sec.$^{-1}$ and the solution stirred at 1000 r.p.m.

Specific first-order rate constants for the oxidation of five different sodium sulfite solutions in the presence of air are shown in Table II. Recrystallization of the sodium sulfite effected a significant change in the rate of oxidation of but one sample. Order of the reaction with respect to oxygen was not experimentally determined. Nevertheless, a very favorable comparison can be made between these data obtained from air oxidation studies and Fuller and Crist's data (10) obtained in a pure oxygen system. Concentration of dissolved oxygen in water in equilibration with the pure gas at atmospheric pressure is approximately five times greater (11, 12) than the concentration of dissolved oxygen in equilibrium with air at atmospheric pressure. Assuming a first-order dependence with respect to dissolved molecular oxygen, the observed rate of reaction in the presence of air should be one-fifth that which is observed in the presence of oxygen. Thus, the specific reaction rate constant in pure oxygen is given by Fuller and Crist as 13×10^{-3} sec.$^{-1}$; one-fifth of this value is 2.6×10^{-3} sec.$^{-1}$, corresponding to the rate constant for the reaction in the presence of air. This latter theoretical value shows close agreement with the average experimental value 2.86×10^{-3} sec.$^{-1}$ reported in Table II.

Inhibitory effect of small amounts of N,N-dimethylformamide (DMF) and N,N-dimethylacetamide (DMA) on sulfite oxidation is shown in Figs. 3 and 4. Oxidation of sulfite is so inhibited in the presence of either 0.01 *M* DMF (0.73 mg. ml.$^{-1}$) or 0.01 *M* DMA (0.87 mg. ml.$^{-1}$) that no detectable reaction takes place within 380 minutes (ten half-lives of the noninhibited reaction). Comparison of inhibitory effects is shown in Table III in which the ratio of the first-order rate for the inhibited reaction, k_2, to the uninhibited reaction, k_1 is presented. The inhibitory effect of these amides on sulfite oxidation may be described with the following equation

$$\frac{-d(S_t)}{dt} = \frac{k_1(S_t)A}{B + m} \qquad \text{(Eq. 1)}$$

where S_t is the total sulfurous acid species, k_1 is the specific rate constant for the uncatalyzed reaction, 2.88×10^{-3} sec.$^{-1}$, m is the molar concentration of additive, and A and B are constants. The form of this equation describing sulfite inhibitors was first employed by Bäckström (13) and later modified by Fuller and Crist (10) in which they found $B = A$ for mannitol inhibition over a 10^5-fold concentration range. Their modification of the empirical inhibitory equation (Eq. 1) has found considerable application in describing sulfite inhibitors (1, 14).

Effect of initial hydrogen ion concentration on the rate and course of sulfite oxidation is shown in Fig. 5. Absolute initial rate of loss of sulfurous acid species, $-d(S_t)/dt$, is very nearly the same over a rather wide range of initial hydrogen ion concentrations as shown in Table IV. The course of the reaction, however, appears to be determined by the initial pH: higher hydrogen ion concentrations decreasing the total amount of sulfurous acid species oxidized during the time period of the experiment.

Table IV.—Absolute Initial Rate of Sulfite Loss and Magnitude of the Catalytic Constant as a Function of Initial pH

pH	$\sqrt{[H^+]} \times 10^4$	$[HSO_3^-] \times 10^{2a}$	k (sec.$^{-1}$) $\times 10^3$	$-d(S_t)/dt \times 10^5$ (M/L.$^{-1}$ sec.$^{-1}$)	$g \times 10^6$ (M/L.)$^{1/2}$ sec^{-1b}
6.95	3.34	1.13	3.2	6.5	2.0
7.15	2.65	0.91	3.4	6.8	2.0
7.45	1.88	0.60	3.3	6.6	2.0
7.80	1.26	0.32	3.1	6.2	2.4
8.20	0.79	0.14	2.7	5.3	2.9

[a] Calculated with the expression $[HSO_3^-] = S_t - SO_3^{2-} = S_t - \frac{S_t}{\left[\frac{[H^+]}{Ka'_2} + 1\right]}$; apparent second ionization constant of sulfurous acid experimentally determined: $K_{HSO_3^-} = 8.4 \times 10^{-8}$. [b] $g = \frac{(-d(S_t)/dt)\sqrt{[H^+]}}{[HSO_3^-]}$.

TABLE V.—EFFECT OF ACIDITY ON REACTION VELOCITY

Initial pH	t(sec.)	S_t × 10^{2a}	$[HSO_3^-]$ × 10^{2b}	$[SO_3^{2-}]$ × 10^2	$[H^+]$ × 10^{8c}
6.95	0	2.00	1.13	0.87	11.2
	30	1.80	1.13	0.67	(14)
	60	1.65	1.13	0.52	(18)
	90	1.50	1.13	0.37	(26)
	120	1.35	1.13	0.22	(42)
	300	1.13	1.13	0.00	3100.
	1200	1.13	1.13	0.00	3100.
7.15	0	2.00	0.91	1.09	7.1
	60	1.60	0.91	0.69	(11)
	120	1.30	0.91	0.39	(19)
	180	1.07	0.90	0.17	(53)
	300	0.80	0.80	0.00	2400.
	1200	0.80	0.80	0.00	2400.
7.45	0	2.00	0.60	1.40	3.6
	60	1.65	0.60	1.05	(4.8)
	180	1.08	0.60	0.48	(11)
	240	0.86	0.60	0.26	(19)
	300	0.74	0.60	0.14	(36)
	600	0.52	0.52	0.00	2100.
	1200	0.52	0.52	0.00	2100.
7.80	0	2.00	0.33	1.67	1.6
	120	1.35	0.33	1.02	(2.7)
	240	0.90	0.31	0.59	(4.4)
	360	0.68	0.30	0.38	(6.6)
	480	0.46	0.30	0.16	(15.7)
	960	0.28	0.28	0.00	1600.
	1200	0.28	0.28	0.00	1600.
8.20	0	2.00	0.14	1.86	0.63
	120	1.40	0.14	1.26	0.95
	240	1.05	0.14	0.91	(1.3)
	480	0.55	0.14	0.41	(2.9)
	720	0.26	0.14	0.12	(9.8)
	1200	0.14	0.14	0.00	1300.
	1500	0.14	0.14	0.00	1300.

[a] $S_t = [HSO_3^-] + [SO_3^{2-}]$. [b] Calcd.: $[HSO_3^-] = S_t - SO_3^{2-} = S_t - \frac{S_t}{\left[\frac{[H^+]}{Ka'_2} + 1\right]}$. Apparent second ionization constant of sulfurous acid; $K_{HSO_3^-}$ 8.2 + 10^{-8}. [c] Experimentally determined $[H^+]$ except for calculated values shown in brackets.

The region at which the rate of oxidation *appears* to be invariant corresponds to conditions where the sulfurous acid species is present almost wholly as hydrogen sulfite, HSO_3^-, as shown in Table V.

At first glance one may intuitively infer that sulfite species are selectively oxidized. This is patently incorrect since it leads to the improbable conclusion that bisulfite species are not air oxidizable. It was pointed out by Abel (9) that the apparent course of the oxidation of mixed sulfurous acid species

$$\begin{array}{r} O_2 + 2HSO_3^- \rightarrow 2HSO_4^- \\ 2SO_3^{2-} + 2HSO_4^- \rightarrow 2HSO_3^- + 2SO_4^{2-} \\ \hline O_2 + 2SO_3^{2-} \rightarrow 2SO_4^{2-} \end{array}$$

is a consequence of the different strengths (12) of the two acids, HSO_3^- and HSO_4^-

$$\frac{K_{HSO_3^-}}{K_{HSO_4^-}} = \frac{[SO_3^{2-}]\,[HSO_4^-]}{[SO_4^{2-}]\,[HSO_3^-]} = \frac{6.24 \times 10^{-8}}{2 \times 10^{-2}} = 3.12 \times 10^{-6}$$

Thus, the oxidation of a solution containing both bisulfite and sulfite ions will always appear to change in total amount of sulfurous acid species, S_t, at the expense of the sulfite species.

The mechanism of sulfurous acid salt oxidation in aqueous systems has been discussed in rigorous detail by Abel (9), and the general aspects of kinetics and mechanism have been the subject of a recent review (1). Abel derived theoretically an expression describing the effect of hydrogen ion concentration on sulfite oxidation

$$-\frac{d(S_t)}{dt} = \frac{g[HSO_3^-]}{\sqrt{[H^+]}} \qquad \text{(Eq. 2)}$$

Validity of this equation was proved by demonstrating that it would describe Fuller and Crist's highly reliable experimental data on sulfite oxidation in presence of pure oxygen (9). Evaluation of g, the catalytic constant, for a pure oxygen system gave the value 8.7×10^{-6} (mole/L.)$^{1/2}$ sec.$^{-1}$ Since this value is proportional to the rate of loss of sulfurous acid species, S_t, and since we may assume a first-order dependency of the rate on dissolved oxygen concentration, air systems in which dissolved oxygen is one-fifth that of pure oxygen systems should yield a value of about 1.8×10^{-6} (mole/L.)$^{1/2}$ sec.$^{-1}$ Experimentally determined values of the constant, g, shown in Table IV appear to stand in reasonable agreement with this value.

The standard oxidation reactor employed in this study appears to provide reproducible and reliable data which are consistent with other carefully done independent studies on sulfite oxidation. Kinetics of the sulfite oxidation process are adequately described by Abel's theoretically derived equations which have received but little attention in pharmaceutical literature. This study corroborates much of Abel's mechanistic interpretations and kinetic expressions.

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Potentiometric Determination of Sulfite Oxidation Rates

By LOUIS C. SCHROETER

Solutions containing hydrogen sulfite, HSO_3^-, and sulfite, SO_3^{2-}, species appear to undergo oxidation at the expense of the divalent anion; this is a consequence of the different strengths of the two acids, HSO_3^- and HSO_4^-. A theoretical equation has been derived which permits calculation of the rate of loss of total sulfurous acid species $[-d(S_t)/dt]$ from a pH-time plot of the oxidizing system. Rapid and reproducible rate studies of oxidizing sulfurous acid salt systems have been made possible through the development of an automated apparatus. Applications of the sensitive radical mediated oxidizing sulfite system for detecting presence of a wide variety of compounds in p.p.m. range are discussed. Empirical equations can be used to obtain semiquantitative analyses of compounds which inhibit the rate of sulfite oxidation in low (10^{-6} M) concentrations.

OXIDATION OF AQUEOUS solutions of sulfurous acid salts in the presence of air proceeds by a free radical chain mechanism; compounds capable of breaking the reaction chain serve as effective inhibitors of the oxidative process (1). Organic compounds in rather dilute solution (10^{-6} to 10^{-4} M) representing the following wide variety of classes are known to inhibit sulfite oxidation: acids (2), alcohols, glycols, mono- and polysaccharides (3–6), amines (7–10), amides (11), aldehydes (2), alkaloids (12), ketones (2), indoles (3), phenols and quinones (13, 14). Inorganic anions such as arsenite, antimonite, phosphite, and cyanide (3, 12) may function as chain terminators; the ammonium cation has been reported to be an effective inhibitor (12). Inhibitory action of these diverse compounds on sulfite oxidation which is now generally interpreted in terms of radical chain termination has been known for well over 60 years as shown by Young's observation (12): "... this inhibitive action is characteristic to a greater or less degree of all organic matter, and, in addition, of many nitrogen compounds not organic."

Most studies of sulfite oxidative behavior have been carried out in a qualitative manner which reflects the difficulty inherent in designing experiments to study free radical solution reactions in a rigorous manner. However, certain investigators, notably Bäckström (4, 6), have exercised greater care in experimental procedures and have treated data mathematically and offered evidence to support mechanisms. Various empirical equations have been used to describe bisulfite inhibition (15); most take the form

$$-\frac{d(S_t)}{dt} = \frac{A}{B + m}\, k_1 \cdot S_t \qquad \text{(Eq. 1)}$$

where S_t is the total sulfurous acid species, k_1 is the specific rate constant for the uncatalyzed reaction, and m is the molar concentration of additive. The constants A and B generally have values the order of 10^{-5} and are specific for each compound or class of compounds although the constants, A and B, are equal (10^{-5}) for the case of mannitol inhibition (5).

The relationship shown in Eq. 1 between the observed rate of reaction for sulfite oxidation and the molar concentration of inhibitor present in solution permits one to determine the concentration of inhibitor by comparing the rate constants for the standard k_1 and the inhibited k_2 reaction

$$\frac{k_2}{k_1} = \frac{A}{B + m} \qquad \text{(Eq. 2)}$$

Application of this equation to the determination of organic substances (m) in solution requires a knowledge of the values of the constants A and B. These constants can be evaluated from rate data obtained in systems containing known amounts of organic compound. However, good approximations may be made by assuming $A = B = 10^{-5}$. The oxidizing sulfite system can thus serve as a unique analytical method for the detection and approximate determination of organic compounds in dilute aqueous solution.

Heavy metal ions are known to function as catalysts of the bisulfite oxidative process. The mechanism proposed (1) for the catalytic effect of copper ions is typical

$$\begin{array}{r} 2(2Cu^{2+} + SO_3^{2-} + H_2O \rightarrow 2Cu^{+} + SO_4^{2-} + 2H^{+}) \\ 4Cu^{+} + O_2 + 4H^{+} \rightarrow 4Cu^{2+} + 2H_2O \\ \hline 2SO_3^{2-} + O_2 \rightarrow 2SO_4^{2-} \end{array}$$

Abel (16) has advanced a strong case for the second sequence as the rate-determining step. The mechanism assumes a steady state production of radicals and a time-dependent reaction with cuprous ion

$$Cu^{+} + OH \rightarrow Cu^{2+} + OH^{-}$$

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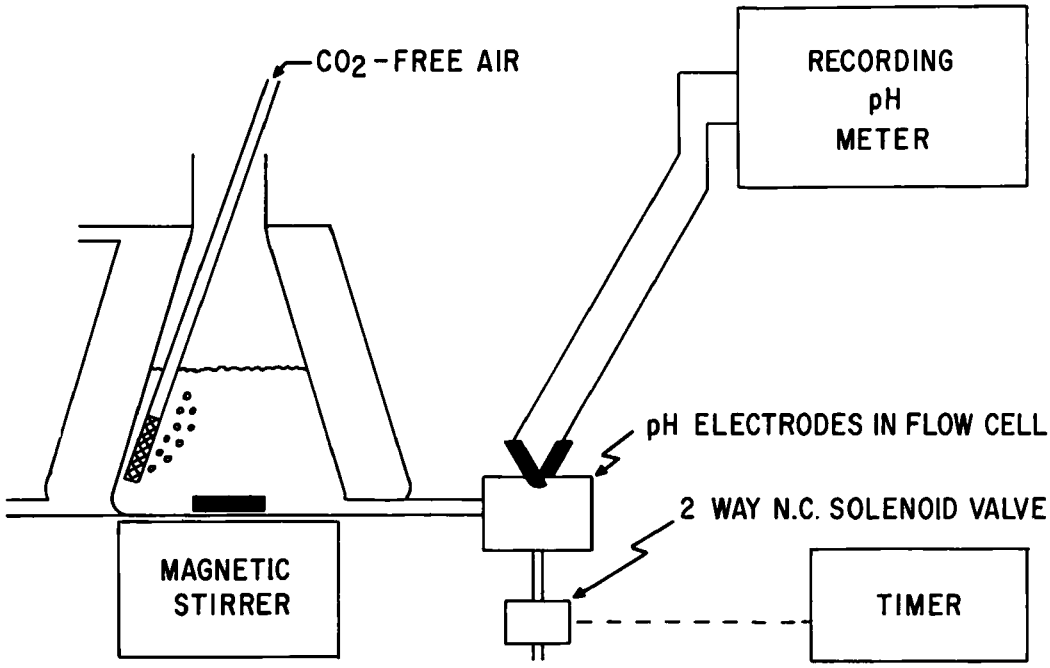

Fig. 1.—Arrangement of oxidation vessel for automatic pH monitoring of samples.

Abel's mechanism is lucid and well documented, but most important, it adequately describes Fuller and Crist's (5) experimental data.

Nonmetal ions may also function as catalysts of the oxidation of sulfurous acid salts. For example, thiosulfate, $S_2O_3^{2-}$, may participate in a chain process with bisulfite and sulfite ions (17). Mechanism of the reaction appears to involve trithionate ion, $S_3O_6^{2-}$, which arises from the interaction of thiosulfate with a OH, O_2^-, or HSO_3 radical

$$S_2O_3^{2-} + SO_3^{2-} + 2OH + 2H^+ \rightarrow S_3O_6^{2-} + 2H_2O$$

The trithionate ion is readily hydrolyzed to thiosulfate and sulfate

$$S_3O_6^{2-} + H_2O \rightarrow S_2O_3^{2-} + SO_4^{2-} + 2H^+$$

resulting in marked catalysis of the oxidative process.

Systematic investigations of the catalytic and inhibitory effect of various substances on the oxidative rate of sulfurous acid salts have been hampered in the past by the difficulty involved in providing reproducible and reliable systems. It is the purpose of this investigation to describe a reasonably simple yet highly reliable experimental technique and automated apparatus for studying the progress of air oxidation of sulfite systems and to demonstrate the analytical potential of these systems for detecting the presence of very low concentrations of organic compounds in aqueous systems.

TABLE I.—RELATIONSHIP BETWEEN INITIAL AND FINAL[a] BISULFITE CONCENTRATION IN OXIDIZED SULFUROUS ACID SALT SYSTEMS

	$[H^+] \times 10^8$	$[HSO_3^-] \times 10^{2b}$	$[SO_3^{2-}] \times 10^{2c}$
Initial	11.2	1.13	0.87
Final	3100	1.13	0.00
Initial	7.1	0.91	1.09
Final	2400	0.80	0.00
Initial	3.6	0.60	1.40
Final	2100	0.52	0.00
Initial	1.6	0.33	1.67
Final	1600	0.28	0.00
Initial	0.63	0.14	1.86
Final	1300	0.14	0.00

[a] "Final" means after 1200 seconds' exposure to standard reaction conditions with no further substantial reaction occurring. *c.f.*: Schroeter, L. C., THIS JOURNAL, 52, 559 (1963). [b] Calculated with the expression

$$[HSO_3^-] = S_t - SO_3^{2-} = S_t - \frac{S_t}{\left(\frac{[H^+]}{K_{a_2}} + 1\right)}$$

Experimentally determined apparent second ionization constant, $K'_{HSO_3^-}$, equals 8.4×10^{-8}. [c] Total sulfurous acid species $[HSO_3^-] + [SO_3^{2-}]$, determined iodometrically.

EXPERIMENTAL

Apparatus and Material.—Air oxidation of sodium sulfite solutions was carried out in a thermostatted vessel of the design shown in Fig. 1. The sampling port was placed tangentially to the vessel wall to take advantage of the centrifugal pumping action imparted to the liquid by the magnetic stirring bar. Samples were periodically removed from the reaction solution and passed through a flow cell containing micro electrodes (glass-calomel); this was done automatically through the use of a timer-controlled solenoid valve.

Air supplied to the reactor was passed through a filter and molecular sieve to remove suspended solid

and liquid particles. The air stream was then conducted through three gas scrubbing towers in series: *a*, sodium hydroxide solution, *b*, sulfuric acid solution; *c*, triple distilled water; gas scrubbers were maintained in a thermostat at the same temperature as the reactor. Flow rates were measured with a calibrated wet gas flow meter[1] and maintained within 5 cm.^{3} sec.$^{-1}$ of the desired flow rate. Tubing was Pyrex glass with short pieces of Tygon used for connections.

Triple distilled water produced from a Pyrex apparatus and stored not longer than 12 hours under nitrogen prior to use was employed for making all solutions and rinsing equipment. Sodium sulfite, reagent grade, was recrystallized twice from distilled water and dried at 90° under vacuum. All other materials were of reagent grade.

The standard reactor was used for all studies. Three-hundred and fifty ml. of sulfite solution was stirred at rates greater than 1000 r.p.m. at 25 ± 0.05° while water-saturated, carbon dioxide-free air was introduced at 50 ± 5 cm.^{3} sec.$^{-1}$ through a medium glass frit beneath the surface of the fluid. The pH of samples removed from the reactor by the automatic sampling system was determined manually with a Beckman GS pH meter, or automatically with a recording Beckman Zeromatic pH meter, or with a Sargent model D titrator. Total sulfurous acid salt concentration of samples was determined iodometrically.

RESULTS AND DISCUSSION

Free radical systems such as oxidizing bisulfite solutions are extraordinarily sensitive to the presence of extremely small amounts of compounds which may act either as inhibitors or catalysts. For example, it was found that certain glass-calomel combination electrodes placed directly in the oxidizing system would not alter or affect the rate of sulfite oxidation while other glass-calomel electrodes caused grossly erratic results. Erratic effects appeared to be related to different inherent diffusion rates of electrolyte solution from the calomel reference electrode. For this reason a pH flow cell was used to measure samples withdrawn from the solution. Measurement of pH was generally made with the more sensitive Beckman GS pH meter; however, the arrangement shown in Fig. 1 where a recording pH meter is employed yields reliable data for most studies.

The relationship between initial and final bisulfite concentration in air-oxidized mixed sulfurous acid species (HSO_3^- and SO_3^{2-}) is shown in Table I. Total sulfurous acid species present in solution, $S_t = HSO_3^- + SO_3^{2-}$, was determined by iodometric titration; concentration of bisulfite species was calculated with

$$[HSO_3^-] = S_t - SO_3^{2-} = S_t - \frac{S_t}{\left[\frac{[H^+]}{Ka'_2} + 1\right]} \quad \text{(Eq. 3)}$$

where Ka'_2, the apparent second ionization constant of sulfurous acid experimentally determined in 0.02 *M* solution at 25°, was 8.4×10^{-8}.

[1] Flowrator—Fischer-Porter Co., Hatboro, Pa.

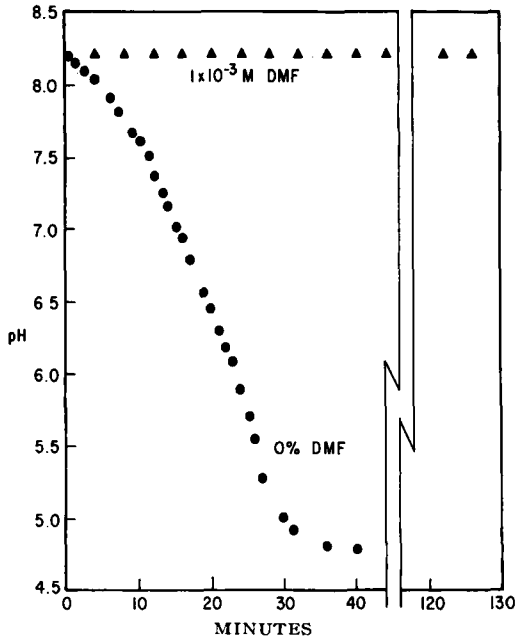

Fig. 2.—The pH of 0.02 *M* sodium sulfite solutions subjected to standard oxidizing conditions at 25°.

The remarkable constancy of the concentration of bisulfite in these systems, $C_{HSO_3^-}$ initial = $C_{HSO_3^-}$ final, is not a consequence of its inability to be oxidized. This reflects the different strengths of the two acids, $K_{HSO_3^-} = 6.24 \times 10^{-8}$, $K_{HSO_4^-} = 2 \times 10^{-2}$.

Solutions containing both bisulfite and sulfite species will always appear to undergo initial rapid oxidation at the expense of the sulfite species

$$\begin{array}{r} HSO_3^- + {}^1/_2O_2 \rightarrow HSO_4^- \\ HSO_4^- + SO_3^{2-} \rightarrow HSO_3^- + SO_4^{2-} \\ \hline SO_3^{2-} + {}^1/_2O_2 \rightarrow SO_4^{2-} \end{array}$$

Thus, total sulfurous acid species, S_t, present in the solution at any time, t, will be approximately described by

$$S_t \cong C_{SO_3^{2-}} + C_{HSO_3^-} \text{ initial} \quad \text{(Eq. 4)}$$

or its equivalent form

$$S_t \cong C_{HSO_3^-} \text{ initial} \left[\frac{Ka'_2}{[H^+]} + 1\right] \quad \text{(Eq. 5)}$$

Using Eq. 5, total sulfurous acid salt concentration may be described by potentiometric determination of hydrogen ion activity if the ionization constant is known; initial bisulfite concentration is determined by material balance and Eq. 3.

The assumption of constancy of bisulfite concentration in air-oxidized systems containing both mono- and di-valent sulfurous acid species is strongly supported by experimental evidence presented in Table I. However, it is important to qualify this assumption by indicating that the final bisulfite concentration refers to conditions such that no substantial change in total sulfurous acid salt concentration takes place within the time period of two half-lives of the initial rapid reaction. Otherwise, the assumption would lead to

the inference that a solution containing only bisulfite species is not subject to oxidation. This is not the case. The lowered rate of oxidation of sulfurous acid systems containing bisulfite, HSO_3^-, as the predominant species is a consequence of the hydrogen ion effect on the rate equation

$$-\frac{d(S_t)}{dt} = \frac{g[HSO_3^-]}{\sqrt{[H^+]}} \quad \text{.(Eq. 6)}$$

where g, the catalytic constant, has the value 2.0×10^{-6} (moles/L.)$^{1/2}$ sec.$^{-1}$ in air-oxidized systems (11). Thus, at pH 4 where bisulfite is the dominant species ($[SO_3^{2-}]/[HSO_3^-] = 6.24 \times 10^{-4}$), the rate of loss of sulfurous acid species, $-d(S_t)/dt$, is at least an order of magnitude less than at pH 8 ($[SO_3^{2-}]/[HSO_3^-] = 6.24$).

Change in pH accompanying oxidation of a solution containing initially 1.86×10^{-2} M sulfite and 0.14×10^{-2} M bisulfite is shown in Fig. 2. The solution containing 1×10^{-3} M N,N-dimethylformamide (DMF) did not change pH when subjected to the standardized oxidation conditions for a period of over 2 hours, nor did the total available sulfurous acid salt concentration (0.02 M) as determined by iodometric titration of samples change during this period. Inhibitory action of DMF has been reported in a prior communication (11).

The solution containing only sulfurous acid species underwent a change in pH from 8.2 to 4.8 as oxidation progressed. Total available sulfurous acid species, S_t, present in solution as a function of time was determined simultaneously by rapid iodometric titration of samples withdrawn from the reaction as shown in Fig. 3 in which solid circles represent experimentally determined values. Reaction profile shows an initial lag time of about 5 minutes due to stirring effects: the volume of solution contained in the reactor was increased

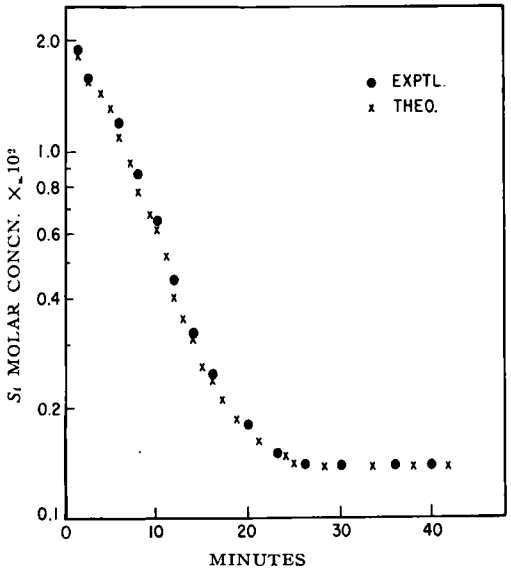

Fig. 3.—Total sulfurous acid salt concn. as a function of time in solution subjected to standard oxidizing conditions. Initial soln. composition: $[HSO_3^-] = 0.14 \times 10^{-2}$ M; $[SO_3^{2-}] = 1.86 \times 10^{-2}$ M

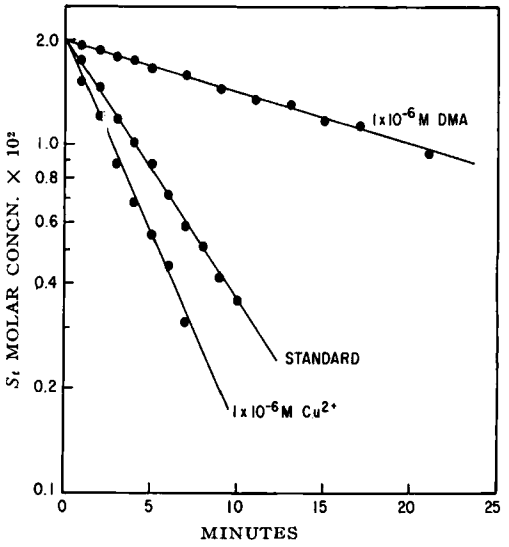

Fig. 4.—Effect of inhibitor (1×10^{-6} M DMA) and catalyst (1×10^{-6} M $CuSO_4$) on rate of sulfite oxidation. Initial solution composition: $[HSO_3^-] = 0.14 \times 10^{-2}$ M; $[SO_3^{2-}] = 1.86 \times 10^{-2}$ M.

from the standard 350 ml. to 450 ml. to illustrate the importance of controlling not only the stirrer speed but also all other factors (volume, vessel geometry, etc.) which may influence effective rates of stirring and dissolution of oxygen.

After the initial 5-minute lag period, the reaction appeared to follow a first-order dependency on total sulfite for about 75% of the reaction with a specific rate constant (2.67×10^{-3} sec.$^{-1}$) comparable to that found in earlier studies (11). As the solution became more acid, the rate of oxidation slowed rapidly and the total sulfurous acid concentration approached the value 0.14×10^{-2} M. Since the pH of the solution at this point was 4.8, this value corresponded almost entirely to bisulfite species. This stands in qualitative support of the assumption that bisulfite concentration appears to remain constant. Theoretical points shown as crosses in Fig. 3 were calculated from potentiometric data shown in Fig. 2 using Eq. 5. Good agreement between experimental and theoretical values attests to the validity of Eq. 5 and the soundness of explicit assumptions upon which it is based.

Analytical application of the automated procedure for detecting the presence of very low concentrations of organic compounds or heavy metal ions is shown in Fig. 4. Apparent first-order plots exhibiting no detectable initial lag periods were obtained under standard operating conditions. Ratio of the specific rate constant, k_2, for the reaction containing 1×10^{-6} M N,N-dimethylacetamide (DMA) to the specific rate constant, k_1, for the standard reaction was calculated

$$\frac{k_2}{k_1} = \frac{5.75 \times 10^{-4} \text{ sec.}^{-1}}{2.80 \times 10^{-3} \text{ sec.}^{-1}} = 0.205.$$

This ratio and reported values (11) for the constants ($A = 0.25 \times 10^{-5}$; $B = 1.0 \times 10^{-5}$) were used in Eq. 2 to calculate the concentration of DMA. The concentration of DMA found by rate data was

2.2×10^{-6} M which agrees within a factor of 2 with the theoretical amount (1×10^{-6} M) present; this appears to be the practical limit of sensitivity of this kinetic analytical system. While the method is semiquantitative, it will detect the presence of many organic compounds in very dilute (10^{-8} to 10^{-6} M) solutions and establish concentrations within an order of magnitude of the true value.

CONCLUSIONS

Radical processes such as oxidizing sulfurous acid salt systems are sensitive to many compounds which act to inhibit or accelerate the chain reaction. Characteristically these compounds are effective in significantly modifying the rate of the oxidation even in extremely high dilutions (10^{-6} M). Rate determinations of uncatalyzed reactions can be made in a matter of minutes with the automated apparatus; rates observed in air oxidized systems can be increased by a factor of 5 by using pure oxygen (Henry's law).

Establishment of a reproducible sulfite-bisulfite system in a standard automated reactor such as described in this report offers a unique detecting system. A wide variety of compounds including acids, alcohols, glycols, polysaccharides, amines, aldehydes, alkaloids, ketones, indoles, phenols and quinones, and *possibly many other classes of organic compounds will measurably inhibit the rate of oxidation in very low concentration.* Likewise, heavy metal ions in parts per million can be detected by observing their catalytic effect on the rate of oxidation. Presence of these various compounds in water or air samples (after bubbling through water) can be readily detected. Semiquantitative relationships between concentration of inhibitor and oxidation rate constant may be established with an empirical equation of the form (Eq. 2).

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The possible role of serotonin in the thyroid action of reserpine was studied in intact rats. Thyroid alteration by chronic reserpine medication and by chronic pharmacological treatment designed to alter serotonin levels and activity were compared with controls by three parameters. Direct effects of reserpine and serotonin were also investigated. Some evidence for reduced thyroid activity by chronic systemic reserpine administration was provided. It was found that reserpine directly *in vitro* slightly increased I^{131} uptake, and that serotonin in direct *in vitro* tests significantly reduced it.

REPORTS of alteration of thyroid function by reserpine have been numerous. Contradictory results, however, in different experimental subjects and in use of different parameters are unexplained, and much information on mechanism is yet to be provided.

Mayer, Kelly, and Morton (1) found that a concentration of 0.083 mg./ml. of reserpine reduced the uptake of I^{131} by calf thyroid slices. Their analysis of slices following incubation indicated that the inhibition of thyroid activity was predominantly one of interference with organic binding of iodine. Ershoff (2) reported that doses of desiccated thyroid and reserpine which were nonlethal when given separately to immature rats resulted in 100% mortality within 2 weeks when given concurrently. DeFelice, *et al.* (3), using oxygen consumption as a parameter of thyroid activity, showed that the administration of reserpine for 5 days to a hyperthyroid guinea pig would lower the oxygen consumption to normal. This group also found that the administration of reserpine to euthyroid animals would lower oxygen

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consumption. Bierwagon (4) reported that a combination of reserpine and thyroxin lowered dehydrogenase activity in thyroid tissue, but that thyrotropin in combination with reserpine prevented this dehydrogenase inhibition. Taylor (5) found that the elevation of cooling rate by reserpine in rats exposed to low temperatures was lowered by prior administration of thyroxin but not by thyrotropin. They believe this confirms that the antithyroid effect is due in part to decreased response of the gland to thyrotropin. Pittman, *et al.* (6), in clinical studies using the thyroid secretion rate as a parameter, found that reserpine reduced thyroid activity in four of nine euthyroid patients. Since this inhibition could not be demonstrated in patients maintained on exogenous thyrotropin, they suggested that reserpine altered thyroid function by diminishing thyrotropin production.

Premachandra (7) reported that no level of reserpine dosage was found (5–100 mcg./100 Gm.) which depressed thyroid function in fowl without simultaneously reducing food intake and thus inferred that thyroid inhibition at high levels of reserpine administration was due to inanition. Uchida (8) concluded from growth studies in chick embryos that reserpine had practically no antithyroid action on the embryonic thyroid.

Elkes (9) found that reserpine counteracted the decrease in thyroid I^{131} uptake induced by LSD (*d*-lysergic acid diethylamide). Results with a similar implication were reported by Kar and Boscott (10) who found that LSD depressed I^{131} uptake in rats *in vivo* and that this depressed uptake was reversed by reserpine. Vogel and Tervooren (11) reported on the basis of oxygen consumption that reserpine reinforced the increased metabolic activity induced by thyroxin in rats. Johnson (12) found that reserpine in doses of 1–4 mg./Kg. increased oxygen consumption in rats.

It is generally recognized that the effects of reserpine are not due to the direct effect of the drug alone but also to release of certain physiological amines. The purpose of this study was to determine the possible role of serotonin in the thyroid action of reserpine by comparing thyroid function alteration induced by reserpine with that observed in animals with pharmacologically altered serotonin levels and activity.

EXPERIMENTAL

Systemic Administration.—Four groups of from 16 to 25 rats each were subjected to the following dosage regimens: (*a*), reserpine, 150 mcg./Kg. daily by intramuscular injection for 28 days; (*b*), LSD, 25 mcg./Kg. daily by subcutaneous injection for 14 days, followed by 50 mcg./Kg. daily by subcutaneous injection for 14 days (dose increased to compensate for tolerance); (*c*), iproniazid, initial single dose of 60 mg./Kg. by subcutaneous injection, followed by 5-hydroxytryptophan (5HTP) 50 mg./Kg. daily for 7 days, followed by 25 mg./Kg. daily for 21 days by intraperitoneal injection (toxicity of original dose level necessitated dosage reduction); (*d*), control (no drug). Rats were healthy Sprague-Dawley males ranging in weight from 350–500 Gm. at the beginning of the experiments.

The following parameters were used to estimate alteration in thyroid function in treated animals and controls: (*a*), basal metabolic rate; (*b*), oxygen uptake of slices of liver and kidney tissue; (*c*), 1 and 2-hour I^{131} uptake by thyroid glands.

Basal metabolic rates (BMR) were calculated from oxygen consumption rate determinations carried out in a modified vacuum desiccator in a temperature-controlled room. Soda lime in the desiccator base and in wire gauze wrapped packs in the upper chamber was used for CO_2 absorption. A 10-ml. pipet attached to the desiccator evacuation outlet was used in the measurement of oxygen consumption. The animals were fasted for 12 hours, placed in the desiccator, and left for a 7-minute temperature equilibration period. Oxygen consumption rates were then determined by a stop watch measurement of the time required for a soap bubble to traverse the 10-ml. interval of the pipet. Means of such determinations were used in calculation of BMR values.

Iodine^{131} uptakes were determined by the method of Williams and Doniach (13). Rats were given 10 μc. of I^{131} by intraperitoneal injection and sacrificed by exsanguination under ether anesthesia at 1 or 2 hours following injection. Thyroids were immediately removed, transferred to planchets, and counted by 1 in. NaI (Th.) crystal scintillation detector. Activities were recorded as percentages of those counts given by a standard sample.

Uptake of oxygen by kidney and liver slices was determined by the direct Warburg manometric method. Tissues taken from animals sacrificed by exsanguination were placed in cold Krebs-Ringer phosphate solution with 0.2% glucose buffered to pH 7.4. Slices were immediately prepared by Stadie-Riggs microtome, blotted with filter paper, and weighed. The gas phase was air. Equilibration period was 10 minutes and readings were made every 15 minutes. Rate of oxygen uptake was determined on wet weight basis and expressed as μl. O_2/100 mg. of tissue per 2 hours.

***In Vitro* Administration.**—Drugs were added directly to flasks containing slices of rat thyroid in determination of direct effects on the gland as measured by I^{131} uptake during incubation. Thyroid donors were healthy Sprague-Dawley males, ranging in weight from 350–450 Gm. and differing in age by no more than 3 weeks. The animals were sacrificed by exsanguination under ether anesthesia. Thyroids were removed, sliced in cold Krebs-Ringer phosphate solution, and placed in 25-ml. flasks containing Krebs-Ringer phosphate to which had been added 0.25 μc. of I^{131} and the drug. Each flask contained the sliced pair of thyroids from a single donor. Incubations were carried out for 2 hours at 38° in a Dubnoff incubator. Six such experiments were carried out, one for each of the following: (*a*),

TABLE I.—BASAL METABOLIC RATE, THYROID I^{131} UPTAKE, AND O_2 UPTAKE BY KIDNEY AND LIVER SLICES FOLLOWING CHRONIC, SYSTEMIC DRUG ADMINISTRATION

Treatment	Dose and Route	BMR ± S.E.[a]	Thyroid I^{131} Uptake ± S.E.[a] (% of Standard) 1 hr.	2 hr.	O_2 Uptake in μl./100 mg. Tissue/2 hr. ± S.E.[b] Kidney	Liver
Reserpine	150 mcg./Kg. daily for 28 days, i.m.	36.14[c] ± 2.04 (12)	2.36 ± 0.133 (11)	4.31[c] ± 0.431 (11)	31.89 ± 1.68 (11)	15.13 ± 0.76 (12)
LSD	25 mcg./Kg. daily for 14 days followed by 50 mcg./Kg. daily for 14 days s.c.	59.01 ± 2.33 (16)	2.21 ± 0.117 (11)	3.76[c] ± 0.367 (12)	25.90 ± 1.02 (10)	15.50 ± 0.36 (13)
Iproniazid and 5HTP	Iproniazid, single dose 60 mg./Kg. s.c., followed by 5HTP, 50 mg./Kg. daily for 7 days, followed by 5HTP 25 mg./Kg. daily for 21 days, i.p.	55.51 ± 3.41 (9)	...	3.60[c] ± 0.487 (7)	23.93 ± 3.31 (5)	8.84[c] ± 0.72 (6)
Control		50.20 ± 2.10 (17)	3.34 ± 0.524 (14)	7.86 ± 1.23 (8)	26.47 ± 1.38 (11)	16.66 ± 0.29 (13)

[a] Numbers in parentheses indicate number of animals. [b] Numbers in parentheses indicate number of flasks. [c] Differs significantly from control at 95% probability.

reserpine, 0.5 mcg./ml.; (*b*), LSD, 1 mcg./L.; (*c*), serotonin, 0.5 mcg./ml.; (*d*), methimazole, 5 mcg./ml.; (*e*), thyrotropin, 0.6 m u./ml.; and (*f*), control (no drug). Concentrations were chosen which would approximate those presented to tissues on systemic administration within usual therapeutic or experimental dosage range. Eleven flasks were incubated in each experiment, ten containing thyroid slices and a standard containing no tissue. Iodine^{131} uptake for each treatment was determined by comparing activities of 0.1-ml. samples from thyroid-containing flasks with the activity of a 0.1-ml. sample taken from the standard flask. These activity differences were expressed as percentages of standard sample counts. Activities were determined by use of a scintillation detector as previously described.

RESULTS

Behavioral effects and other observable changes were recorded during the 28-day periods of systemic drug administration. Reserpine-treated animals began to present signs of decreased activity after 2 or 3 days of drug administration. Ptosis was observed in all reserpinized animals beginning during the first week and continuing through the course of the experiment. Animals receiving LSD did not demonstrate signs of extreme behavior alteration; however, there were occasional signs of erratic activity such as backing movements and purposeless activity usually occurring within 20–30 minutes after injection. Animals receiving 5HTP following iproniazid presented evidence of drug toxicity in lethargy, anorexia, and weight loss. Four animals in this group died during the first week of treatment. this lethality necessitated a reduction in dose. Signs of toxicity persisted, however, and at sacrifice, kidneys of all animals in this group were found to be reduced in size and altered in color and consistency. Microscopic examination of stained sections showed tubular damage. Because of loss of animals during the dosing period, I^{131} uptakes were limited to 2-hour determinations for this group.

Table I presents data for BMR, I^{131} uptake, and tissue slice oxygen uptake for the four groups of systemically treated rats. Table II presents data from measurement of direct effect of the several treatments on thyroid tissue slices.

TABLE II.—I^{131} UPTAKE BY THYROID SLICES ON INCUBATION WITH CERTAIN DRUGS

Drug	Concentration	Flasks, No.	Uptake %, ± S.E.
Reserpine	0.5 mcg./ml.	10	47.4[a] ± 2.1
Serotonin	0.5 mcg./ml.	10	9.4[a] ± 1.7
LSD	1 mcg./L.	10	12.0[a] ± 2.0
Methimazole	5 mcg./ml.	10	0[a]
Thyrotropin	0.6 m u./ml.	10	35.0 ± 3.3
Control	...	10	30.4 ± 6.6

[a] Differs significantly from control at 95% probability.

Two of the parameters, BMR and 2-hour I^{131} uptake, gave evidence of a decreased thyroid activity in response to chronic reserpine administration. In the direct *in vitro* tests, however, reserpine increased I^{131} uptake by thyroid slices. This increased uptake was found to be significant at 95% probability when compared with uptakes of both the control and 0.6 m u./ml. of thyrotropin.

Increasing serotonin level by chronic administration of 5HTP following an initial iproniazid injection (14, 15) failed to alter BMR and oxygen consumption of isolated kidney slices but did reduce significantly the uptake of I^{131} at 2 hours and reduced the consumption of oxygen by liver slices. Serotonin added directly to incubating thyroid slices also significantly reduced I^{131} uptake.

Rats given LSD for 28 days showed a reduced 2-hour thyroid I^{131} uptake, but BMR values and oxygen uptakes by kidney and liver slices were not significantly different from controls. The direct effect of LSD on thyroid slices was a decrease in uptake of I^{131}.

DISCUSSION

Some evidence of a reduced rat thyroid activity in response to chronic reserpine administration was provided by reduction in both BMR and 2-hour I^{131} uptake. However, failure of reserpine to reduce I^{131} uptake of incubating thyroid slices when added directly to the medium suggested that the effects of reserpine on the thyroid were not direct. Since reserpine releases serotonin from binding sites and is believed to effect many of its actions through this medium, the direct effect of serotonin on thyroid slice I^{131} uptake was measured. Results of this

experiment indicated a significant capacity on the part of serotonin to reduce thyroid activity. This suggests that the thyroid effects of chronically administered reserpine are accomplished through the intermediation of serotonin. If this were true it might be expected that LSD through serotonin antagonism (16, 17) would increase thyroid activity, and that an increase in serotonin blood level induced by monoamine oxidase inhibition and 5HTP would reduce thyroid activity. However, the data obtained in testing these assumptions were not conclusive or consistent. There are possible explanations for failure of these attempts at confirmation. That both LSD and serotonin decreased I^{131} uptake by thyroid on *in vitro* administration may be only further evidence for the lack of specific antagonism between the two. In evaluating the role of free serotonin maintained in excess as presumably was accomplished by use of the drug combination, the possibility that toxicity may have altered parameters must be considered.

The evidence for a thyroid inhibiting effect for LSD is consistent with results obtained by others (9, 10). The significance of this in relation to the reserpine mechanism is not apparent. The finding that reserpine *in vitro* significantly increased I^{131} uptake by thyroid slices was not in agreement with Mayer, *et al.* (1), who used calf thyroid and a considerably higher concentration of reserpine. This phase of the work will receive further attention as also will the lethality of the combined effects of iproniazid and 5HTP.

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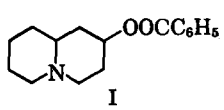
Local Anesthetic Esters Derived from 2-Hydroxyquinolizidine

By CHANDULAL N. PATEL and TAITO O. SOINE

Several pairs of substituted benzoate esters derived from 2-hydroxyquinolizidine and 3-(2-methylpiperidino)propanol were synthesized in order to enable comparison of each pair for relative duration of local anesthetic activity in the rabbit cornea. The tests indicate, in general, the 2-hydroxyquinolizidine esters provided a greater duration of activity than the corresponding esters of 3-(2-methylpiperidino)-propanol. Some of the implications of this increase in duration of action with respect to the structures are discussed.

RHODES AND SOINE (1), in 1956, reported the synthesis of a series of 2-hydroxyquinolizidine esters specifically designed as anticholinergics. The selection of 2-hydroxyquinolizidine as the aminoalcohol was based on its formal relationship to tropine with respect to the relative positions of the amine and alcohol functions. No mention was made of local anesthetic activity although the relationship of 2-hydroxyquinolizidine to ecgonine would be analogous. In 1960, Counsell and Soine (2) described the preparation of selected esters of 1-, 2-, and 3-hydroxyquinolizidines and (among the activities reported) noted local anesthetic activity associated with most of their esters. In connection with the comparative local anesthetic activities, two observations were of particular significance with respect to the present report: *a*, the activity of 2-hydroxyquinolizidine benzoate (I) was 3 and 5 times greater, respectively, than the activities of the isomeric 1- and 3-hydroxyquinolizidine benzoates, and *b*, the activity of I was 1.72 times greater than that of piperocaine (II) which was used as the standard for compari-



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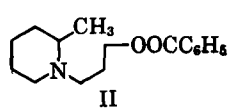

II

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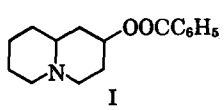

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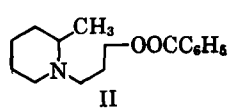

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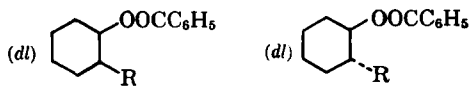
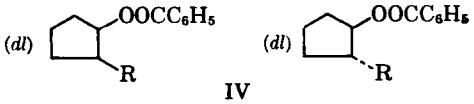
son. The latter observation was of interest because I could be looked upon as a closed ring analog of II. It was of further interest to note that the 2-methyl group in the piperidine moiety of II seems to have a direct influence upon activity inasmuch as its absence leads to inactivity (3). If one considers that the 2-methyl group of II imposes a degree of steric restriction to the esterified alkyl chain attached to the nitrogen of II it is immediately apparent that the closure of the esterified alkyl chain into a ring system as in I imposes still further steric restriction. That steric requirements for local anesthetic activity may be of importance is illustrated by several observations. Among these may be cited the findings of Mannich, *et al.* (4), with respect to the differing activities of the *cis* and *trans* isomers of III and IV wherein one form



III



IV

(R = —$CH_2N(CH_3)_2$)

(not specified) possesses strong activity and the other has either no activity or a greatly lessened activity. Likewise, the recent findings of Nachmansohn in connection with his demonstrations of the chemical basis of nerve activity (5) would seem to imply that structural specificity is important in interaction of the anesthetic base with receptor protein. Apparently, however, the structural specificity does not encompass optical isomers inasmuch as these have been shown in virtually all cases to be of essentially the same activity. Another factor in enhanced activity may be envisioned because esters of secondary alcohols, for steric reasons, are less rapidly hydrolyzed than corresponding esters of primary alcohols which would be expected *a priori* when comparing I and II.

These observations have led us to attempt to determine whether an enhanced duration of anesthetic activity of the 2-hydroxyquinolizidine-type esters over the 3-(2-methylpiperidino)-propanol esters was a general trend. To accomplish this purpose it was necessary to synthesize not only selected esters of the former but also the corresponding esters of the latter for direct comparison. The esters to be synthesized were selected on the basis of the previously reported activities by McElvain and Carney (6) of the 3-(2-methylpiperidino)propanol esters. The rabbit cornea as utilized by McElvain and Carney was the test object in the present study.

DISCUSSION

The synthetic method employed for 2-hydroxyquinolizidine was essentially that of Counsell and Soine (2) with incorporation of certain modifications in the synthesis of the intermediate ethyl 2-pyridyl acetate as described by Winterfeld and Flick (7). 3-(2-Methylpiperidino)propanol was synthesized by the method of McElvain and Carney (6). The necessary methyl esters were purchased where possible or synthesized by standard methods wherever necessary. The synthesis of esters of both aminoalcohols was carried out most conveniently by the ester-interchange method as described by Counsell and Soine (2). This method, for benzoate esters, has proved to be superior to the usual method of interacting an acid chloride with the aminoalcohol. All esters were prepared as hydrochlorides and crystallized to constant melting points. In general, it was found that the ester hydrochlorides derived from 2-hydroxyquinolizidine were less water soluble than the corresponding esters derived from 3-(2-methylpiperidino)propanol. Indeed, it was found impossible to carry out suitable anesthetic tests on several of the 2-hydroxyquinolizidine ester hydrochlorides for this reason.

Testing of the esters for their comparative anesthetic duration times was accomplished by a comparison of the corneal anesthetic duration time ratios on several rabbits. The methods employed are described in the experimental section. The results show conclusively that there is a significant enhancement of the duration of corneal anesthesia by using 2-hydroxyquinolizidine as the esterified aminoalcohol rather than 3-(2-methylpiperidino)-propanol. The average enhancement ratio appears to be approximately 1.40 with a variation from a low value of 1.09 (*o*-hydroxybenzoate) to a high value of 2.38 (*o*-*n*-butoxybenzoate). It should be pointed out that this high value was the only one of its magnitude with the exception of the *o*-benzoylbenzoate which was 2.12. Most of the values were clustered closely around a narrower range centering on 1.30.

The reasons for the enhancement of duration can be speculated upon only. The steric factor has already been mentioned, and it is entirely possible that a better fit at the receptor site is obtainable with molecules that are relatively more fixed sterically than with more flexible molecules. Also one can reasonably assume two major mechanisms whereby the activity of a local anesthetic ester can be terminated. The first of these would be by diffusion from the site of action and the second would be enzymatic inactivation through esterase hydrolysis. Both processes can be assumed to operate at any one site of activity but probably influence the disposal of the ester in varying proportions depending on the particular locus of action. In the present instance, if one assumes that enzymatic activity has at least some role in the disposal of the ester, then it becomes reasonable to assume that steric inhibition to hydrolysis should lead to greater duration of activity. Such steric

TABLE I.—SYNTHESIZED METHYL BENZOATES

Benzoic Acid from Which Derived	Formula	Method of Prepn.	M.p., °C. or B.p., °C., (mm. Hg)	Yield, %	Analysis[a] —Carbon— Calcd.	Found	—Hydrogen— Calcd.	Found	Ref.
p-Phenyl	$C_{14}H_{12}O_2$	2	117–118	93	...	...	...	...	(18)
o-(*n*-Butoxy)	$C_{12}H_{16}O_3$	1	130–132 (6)	63	...	...	...	...	(19, 20)
p-(*n*-Butoxy)	$C_{12}H_{16}O_3$	1	105–110 (0.2)	61	...	...	...	...	(12)
p-iso-Butoxy	$C_{12}H_{16}O_3$	1	110–115 (0.4)	24	69.20	68.96	7.74	7.83	...
p-*sec*-Butoxy	$C_{12}H_{16}O_3$	1	95–100 (0.25)	53	69.20	68.96	7.74	7.77	...
p-iso-Amyloxy	$C_{13}H_{18}O_3$	1	124–125 (1.4)	63	...	...	...	...	(19)
p-(*n*-Hexyloxy)	$C_{14}H_{20}O_3$	1	132–137 (0.5)	59	71.15	71.25	8.53	8.59	...
p-Phenoxy	$C_{14}H_{12}O_3$	2	60	81	...	...	...	...	(14)
p-Benzyloxy	$C_{15}H_{14}O_3$	1	99	74	...	...	...	...	(19)
p-(4-Methoxyphenyl)	$C_{15}H_{14}O_3$	2	172–174	86	...	...	...	...	(16)

[a] New compounds.

inhibition combined with a possible better receptor fit may account for the observed longer duration of activity of the hydroxyquinolizidine esters in the present study.

EXPERIMENTAL

2 - Hydroxyquinolizidine.—*2 - Pyridylacetonitrile.*—This compound was prepared according to the method of Winterfeld and Flick (7) in 77.4% yield as a light yellow oil, b.p. 95–97° (3.7 mm.), n_D^{29} 1.5192 reported, b.p. 79–81° (0.4 mm.)(7); b.p. 80–85° (0.5 mm.), n_D^{29} 1.5193 (8); n_D^{25} 1.5224 (9). A picrate was formed in and recrystallized from absoute ethanol, m.p. 160–162° (reported, m.p. 155–157° (9)).

Ethyl 2-Pyridylacetate.—It was prepared according to the Winterfeld and Flick procedure (7) in a yield of 75.1% as a greenish-yellow liquid, b.p. 90–91° (0.3 mm.), n_D^{29} 1.4921. These properties, together with the picrate, m.p. 137–138°, corresponded in close detail to those previously reported in the literature (1, 10, 11).

2-Hydroxyquinolizidine.—Using ethyl 2-pyridyl acetate as the starting material, the synthesis of this aminoalcohol was carried out essentially as described by Rhodes and Soine (1) employing modifications suggested by Counsell and Soine (2). The yields of all intermediates and of the final product closely approximated those found by the above authors.

3-(2-Methylpiperidino)propanol.—This aminoalcohol was prepared from 2-methylpiperidine and trimethylene chlorohydrin according to the general procedure outlined by McElvain and Carney (6) in 60% yield, b.p. 110–115° (10 mm.) (reported, b.p. 112° (15 mm.) (6)).

Methyl Benzoates (see Table I).—Methyl benzoate, ethyl *o*-benzoylbenzoate, methyl *p*-(*n*-amyloxy)-benzoate and methyl *o*-hydroxybenzoate were purchased from commercial sources. The following examples describe the methods that were used for the synthesis of the other esters.

Alkoxybenzoates.—These were prepared by two methods: *1*, the methyl ester of the phenolic acid was alkylated with the appropriate alkyl halide in absolute methanol in the presence of sodium methoxide, and *2*, the appropriate benzoic acid was esterified with methanol and sulfuric acid. These are illustrated by the following examples.

Methyl p-(n-Butoxy)benzoate.—Sodium (4.6 Gm., 0.2 mole) was dissolved in 100 ml. of absolute methanol. Methyl *p*-hydroxybenzoate (30.4 Gm., 0.2 mole) and 36.8 Gm. (0.2 mole) of *n*-butyl iodide were added. The mixture was refluxed for 12 hours, cooled, and filtered free of inorganic salts. About half of the methanol was distilled off under reduced pressure, and the precipitated inorganic salts were removed by filtration. The solution was rendered alkaline with 5 ml. of 10% sodium hydroxide solution to dissolve unaltered methyl *p*-hydroxybenzoate, and the mixture was treated with 100 ml. of ether and 50 ml. of water. The ether layer and second ether extract were combined. The ether extracts were dried over anhydrous sodium sulfate and, after removal of solvent, the product was distilled to give 25.4 Gm. (61%) of product; b.p. 105–110° (0.2 mm.) (reported, b.p. 190–195° (20 mm.)(12)).

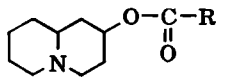
Methyl p-Cyclohexyloxybenzoate.—*p*-Cyclohexyloxybenzoic acid[1] (5 Gm.) absolute methanol (75 ml.), and 7.5 ml. of concentrated sulfuric acid were combined in a 100-ml. round-bottom flask and gently refluxed for 2 hours. The excess of methanol was removed under reduced pressure and the red-colored residue was treated with water. The insoluble ester was extracted from the aqueous mixture with ether and the ether washed with 5% aqueous sodium bicarbonate solution. The ether layer was dried over anhydrous sodium sulfate and after removal of the solvent, provided 3.5 Gm. of an orange-colored liquid. Attempts to distill this material resulted in decompositon and, therefore, it was used in the crude state for further synthesis for which it proved satisfactory.

Miscellaneous Benzoates.—*Methyl p-Phenoxybenzoate.*—A solution of 52.8 Gm. (0.335 mole) of potassium permanganate in 1500 ml. of water was added to a mixture of 20 Gm. (0.0704 mole) of 3-(*p*-phenoxybenzoyl)-propionic acid[2] and 20 Gm. (0.357 mole) of potassium hydroxide in 200 ml. of water. The mixture was heated on a steam bath for 5 hours. At the end of this time the mixture was cooled and carefully acidified with sulfuric acid. The mixture was heated for 30 minutes and cooled. The precipitated manganese dioxide was removed by the addition of a slight excess of 10% sodium bisulfite solution. The precipitated acid was removed by filtration and recrystallized from ethanol; yield, 14.0 Gm. (92.9%), m.p. 159–160° (reported, m.p. 160° (13)).

[1] The authors express their appreciation to Eli Lilly and Co., Indianapolis, Ind., for a generous gift of *p*-cyclohexyloxybenzoic acid.

[2] Distillation Products Industries, Rochester, N. Y.

TABLE II.—ESTERS OF 2-HYDROXYQUINOLIZIDINE



No.	R	Recrystallized from[a]	M.p., °C.	Molecular Formula	Analysis: Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
I	—C_6H_5	A	265–266	$C_{16}H_{21}NO_2 \cdot HCl$	64.96	65.17	7.50	7.54
II	—$C_6H_4C_6H_5(p)$	B	283–285 dec.	$C_{22}H_{25}NO_2 \cdot HCl \cdot {}^1/_2\text{-}C_2H_5OH$	69.94	69.78	7.40	7.32
III	—$C_6H_4OH(o)$	C	238–239	$C_{16}H_{21}NO_3 \cdot HCl$	61.63	61.58	7.11	7.19
IV	—$C_6H_4O(CH_2)_3CH_3(o)$	D	171–172	$C_{20}H_{29}NO_3 \cdot HCl$	65.29	64.75	8.22	8.22
V	—$C_6H_4O(CH_2)_3CH_3(p)$	A	195–197	$C_{16}H_{21}NO_3 \cdot HCl$	65.29	65.19	8.22	8.20
VI	—$C_6H_4OCH_2CH(CH_3)_2(p)$	D	221–222	$C_{20}H_{29}NO_3 \cdot HCl$	65.29	65.02	8.22	8.14
VII	—$C_6H_4OCH(CH_3)C_2H_5(p)$	A	192–193	$C_{20}H_{29}NO_3 \cdot HCl$	65.29	64.89	8.22	8.22
VIII	—$C_6H_4O(CH_2)_4CH_3(p)$	A	198–199	$C_{21}H_{31}NO_3 \cdot HCl$	66.04	65.65	8.44	8.60
IX	—$C_6H_4O(CH_2)_2CH(CH_3)_2(p)$	D	217–218	$C_{21}H_{31}NO_3 \cdot HCl$	66.04	66.04	8.44	8.45
X	—$C_6H_4O(CH_2)_5CH_3(p)$	A	193–194	$C_{21}H_{31}NO_3 \cdot HCl$	66.73	66.52	8.66	8.59
XI	—$C_6H_4OCH(CH_2)_4CH_2(p)$	D	213–215	$C_{22}H_{31}NO_3 \cdot HCl$	67.07	66.57	8.19	8.19
XII	—$C_6H_4OC_6H_5(p)$	A, F	197–198	$C_{22}H_{25}NO_3 \cdot HCl$	68.12	67.85	6.76	6.70
XIII	—$C_6H_4OCH_2C_6H_5(p)$	B	265–267	$C_{23}H_{27}NO_3 \cdot HCl$	68.72	68.56	7.02	7.07
XIV	—$C_6H_4COC_6H_5(o)$	D	194–195	$C_{23}H_{25}NO_3 \cdot HCl$	69.07	69.11	6.55	6.54
XV	—$C_6H_4C_6H_4OCH_3(pp')$	B	276–278	$C_{23}H_{27}NO_3 \cdot HCl$	68.73	68.72	7.02	7.34

[a] A, Ethanol, isopropyl ether; B, ethanol; C, absolute ethanol; D, methylene chloride, ethyl acetate; E, ethyl acetate, ether; F, isopropanol, ether.

The esterification of the phenoxybenzoic acid was carried out essentially as described above for *p*-cyclohexyloxybenzoic acid (*q.v.*) to yield 6.5 Gm. (81%) of colorless crystals after recrystallization from a methanol-water mixture, m.p. 60° (reported, m.p. 59.5 to 60° (14)).

Methyl p-(4-Methoxyphenyl)benzoate.—The first step in this synthesis was the preparation of *p*-(4-methoxyphenyl)acetophenone according to the procedure of Johnson, *et al.* (15). The product was prepared in a 53% yield, m.p. 145–147° and following recrystallization gave pure material, m.p. 153–154° (reported, m.p. 153–154° (15)).

The above product was then oxidized as described by Fieser and Bardher (16) with permanganate to provide a 56% yield of *p*-(4-methoxyphenyl)benzoic acid, m.p. 248–249° (m.p. reported 248–249° (16)).

The above acid was esterified with methanol and sulfuric acid in the manner described above for alkoxybenzoates to give a product, recrystallized from methanol, m.p. 172–174° (reported, m.p. 172–173° (16)). The yield was 86%.

2-Hydroxyquinolizidine Esters.—These esters were all synthesized by the ester-interchange method as described by Counsell and Soine as Method 2 (2). The analytical and physical constants are recorded in Table II.

3-(2-Methylpiperidino)propanol Esters.—These esters were also synthesized by the ester-interchange method according to the above method of Counsell and Soine (2). They all agreed within a few degrees with the reported melting points of McElvain and Carney (6) and from the constancy of their melting points were assumed to be identical with the products of the former workers and of a suitable purity for pharmacological testing.

Pharmacological Testing

Solutions.—All of the salts were dissolved in distilled water in identical concentrations for each pair of compounds tested. The solutions were made on the day of use in every case. These solutions represented essentially equimolar concentrations because of the negligible difference in molecular weights between each pair. The hydrochlorides of *p*-phenylbenzoic, *p*-(4-methoxyphenyl)benzoic, *p*-phenoxybenzoic, and *p*-benzyloxybenzoic acid esters were too insoluble to allow preparation of solutions at the concentration required and thus were not included in the comparison studies.

TABLE III.—LOCAL ANESTHETIC DURATION COMPARISONS

Ester Pair Corresponding to the Esters as Numbered in Table II.	Concentration, %	Duration,[a] min. A	Duration,[a] min. B	Duration Ratio[b] A/B
I	1.00	14	9	1.54 (±0.29)
III	1.00	34	32	1.09 (±0.11)
IV	0.30	38	16	2.38 (±0.36)
VII	0.25	43	35	1.25 (±0.06)
XIV	0.20	58	29	2.03 (±0.63)
X	0.10	24	18	1.29 (±0.09)
XI	0.10	26	21	1.24 (±0.08)
VI	0.10	29	23	1.25 (±0.10)
IX	0.10	31	24	1.32 (±0.33)
V	0.10	53	43	1.28 (±0.13)
VIII	0.10	58	40	1.44 (±0.17)

[a] A = 2-Hydroxyquinolizidine esters. B = 3-(2-methylpiperidino)propanol esters. [b] 95% confidence limits.

Animals.—Young adult white rabbits, equal numbers of each sex, were used for the corneal testing. The eyelids were carefully trimmed to remove the eyelashes. In most tests they were used only once each day but on occasion were tested in the morning and again in the afternoon.

Test Procedure.—The usual method of instillation for corneal testing was employed in which 0.5 ml. of solution was introduced into the conjunctival sac from a blunt-tipped syringe and allowed to act upon the cornea for 1 minute before beginning the duration count. All compounds induced anesthesia within the 1-minute period as evidenced by the absence of the wink reflex when the cornea was stimulated with a blunt glass rod. The time re-

quired for the animal to regain the wink reflex when the stimulus was applied was recorded in minutes. In most cases six rabbits were used for each comparison although in a few cases five were used and in one case 12 were employed. Each pair of esters was tested on the same rabbit's eyes with the right eye being used for one-half of the tests on the hydroxyquinolizidine esters and the left eye for the other half. Concomitantly, the alternate eye of the rabbits was used for the other ester. At no time were both eyes subjected to anesthesia at the same time.

The duration ratio between the pairs of compounds in each animal was obtained by dividing the duration of the 2-hydroxyquinolizidine ester by that of the 3-(2-methylpiperidino)propanol ester. The mean of these ratios was determined and the 95% confidence limits of the mean were calculated assuming a normal distribution for the mean and a chi-square distribution for the squares of the deviations (17). These results are given in Table III.

Observations.—No irritation of the cornea was observed with either series of esters in the concentrations employed. Both the *p*-isoamyloxybenzoate and the *o*-benzoylbenzoate were erratic in the 2-hydroxyquinolizidine series with respect to the comparative durations. The latter, in particular, exhibited an unpredictable behavior with the duration ratios varying widely (0.83 to 3.86). For this reason, the 95% confidence interval varies more widely than any of the other pairs tested. A further observation of interest was that the 2-hydroxyquinolizidine esters induced much less xerophthalmia than the corresponding 3-(2-methylpiperidino)-propanol esters.

SUMMARY

1. Fifteen new substituted benzoate esters of 2-hydroxyquinolizidine have been synthesized and described because of an interest in their comparative local anesthetic activity with the corresponding esters of 3-(2-methylpiperidino)propanol.

2. Four new methyl esters of substituted benzoic acids have been synthesized and described in the course of the investigation.

3. Several of the newly synthesized esters have been compared with their counterparts obtained from the esterification of 3-(2-methylpiperidino)-propanol for duration of corneal anesthesia in the rabbit. The observed results indicate that an enhanced duration of action can be expected as a general trend when comparing the two esters derived from the same acid. The duration ratios varied from 1.09 (± 0.11) for the *o*-hydroxybenzoates to 2.38 (± 0.36) for the *o*-(*n*-butoxy)benzoates.

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RECENT STUDIES by Morozowich, *et al.* (1), on the release of benzphetamine from pellets of benzphetamine pamoate in 0.12 *N* hydrochloric acid medium led these investigators to propose that in this instance the rate was controlled by a layer of pamoic acid which was deposited on the pellet surface according to the reaction:

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This suggestion was based upon the reality of the pamoic acid layer determined by assay of the pellets exposed to the solution and the relative small effect of agitation on the rate of benzphetamine release.

It appeared worthwhile to examine this problem further, both experimentally and theoretically, particularly since the basic considerations may apply to other situations.[1] The results of a detailed analysis of this problem are presented in this report.

THEORY

The Model and Assumptions.—We will assume that the above picture is correct, *i.e.*, the rate of benzphetamine release is controlled by diffusional processes in the pamoic acid layer. Our concern will be only for the case in which the dissolving medium is acidic.

The model is illustrated in Fig. 1. At some time after the pellet is placed in the acid solution, a layer of pamoic acid of thickness s has formed on the surface. The H^+ must diffuse through this layer and react with the pamoate ion to precipitate more acid, releasing the benzphetamine cation

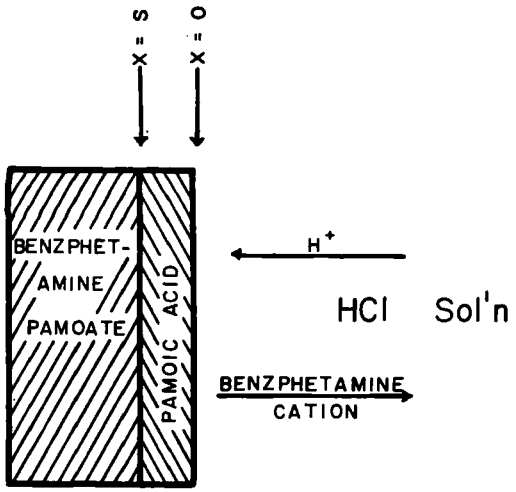

Fig. 1.—Model for the release of benzphetamine from benzphetamine pamoate in hydrochloric acid solution. See text for explanation.

which then diffuses out through the layer. Some of the benzphetamine cation will diffuse out without being released by the H^+ since benzphetamine pamoate has a finite solubility.

In order to make this problem mathematically tractable it is necessary to incorporate the following assumptions into the model: (*a*) quasi-steady-state in the pamoic acid layer; (*b*) Fick's law of diffusion is obeyed, constant effective diffusion coefficients in the layer, and diffusion takes place only in the aqueous phases of the layer; (*c*) effects of liquid diffusion layer negligible; (*d*) initial non-steady-state effects negligible. Some of these assumptions will be examined later.

Concentration-Equilibria-Diffusion Relations.—Let us now obtain an expression for the instantaneous release rate of benzphetamine at a given pamoic acid layer thickness s with the assumption that steady state concentration gradients are present. The appropriate boundary conditions for the problem are $C_{H^+} = C^\circ_{H^+}$, $C_{B^+} = C^\circ_{B^+}$, $C_{P=} = C^\circ_{P=}$, and $C_{H_2P} = K_l$ = constant at $x = 0$ where C_{H^+}, C_{B^+}, $C_{P=}$, and C_{H_2P} are the H^+, benzphetamine-H^+, pamoate anion, and the pamoic acid (in solution) concentrations, respectively. At $x = s$ we have $C_{H^+} = C^s_{H^+}$, $C_{B^+} = C^s_{B^+}$, $C_{P=} = C^s_{P=}$, and $C_{H_2P} = K_l$.

As before (4) we may write down the appropriate differential equations for diffusion and simultaneous chemical reaction in a one dimensional situation. Throughout the region $0 \leq x \leq s$ we have

$$D_{P=}\left(\frac{d^2C_{P=}}{dx^2}\right) + \Phi = 0 \qquad \text{(Eq. 1)}$$

$$D_{H^+}\left(\frac{d^2C_{H^+}}{dx^2}\right) + 2\Phi = 0 \qquad \text{(Eq. 2)}$$

$$D_{B^+}\left(\frac{d^2C_{B^+}}{dx^2}\right) = 0 \qquad \text{(Eq. 3)}$$

A forth equation involving H_2P is omitted because $C_{H_2P} = K_l$ is independent of x as long as solid pamoic acid is present. In Eqs. 1 to 3 the D's are the effective diffusion coefficients for the species indicated and Φ is the rate of reaction per unit volume for the reaction $P^= + 2H^+ = H_2P$ (aq.) = H_2P (s). Since there will be no formation or disappearance of benzphetamine in the pamoic acid layer, the second derivative of C_{B^+} is always zero.

If Eqs. 1 and 2 are combined and integrated over the limit $x = 0$ to $x = s$, we obtain

$$G = \frac{2D_{P=}(C^s_{P=} - C^\circ_{P=}) + D_{H^+}(C^\circ_{H^+} - C^s_{H^+})}{s} \qquad \text{(Eq. 4)}$$

Furthermore, integration of Eq. 3 over $x = 0$ to $x = s$ gives

$$G = \frac{D_{B^+}(C^s_{B^+} - C^\circ_{B^+})}{s} \qquad \text{(Eq. 5)}$$

In these equations, G is the rate of release of benzphetamine per unit area of pellet surface.

In order to eliminate the unknowns, $C^s_{P=}$, $C^s_{H^+}$, and $C^s_{B^+}$ from the expression for G, it is necessary to introduce the appropriate equilibrium relationships. These are the solubility product expressions for benzphetamine pamoate

$$K_{sp} = C^2_{B^+}C_{P=} \qquad \text{(Eq. 6)}$$

and the dissociation constant expression for pamoic acid

$$K_A = \frac{C^2_{H^+}C_{P=}}{C_{H_2P}} = \frac{C^2_{H^+}C_{P=}}{K_l} \qquad \text{(Eq. 7)}$$

If Eqs. 4–7 are combined, we may obtain the following relation upon eliminating G

[1] A similar but simpler situation is that of drug release from emulsion ointment bases (2). Another analogous situation has been reported by Levy and Procknal (3) for the release of acetylsalicylic acid from aluminum acetylsalicylic acid pellets in a basic aqueous medium which involves the formation of a water-insoluble aluminum compound on the surface of the pellets.

$$C^{s}_{B^+} = \frac{D_{B^+} C^{\circ}_{B^+} + 2D_{P=} [(K_{sp}/C^{s2}_{B^+}) - (K_A K_l / C^{\circ 2}_{H^+})] + D_{H^+} C^{\circ}_{H^+}}{D_{B^+} + D_{H^+} (K_A K_l / K_{sp})^{1/2}} \quad \text{(Eq. 8)}$$

Now the numerical values[2] for K_{sp}, K_A, and K_l are $K_{sp} = 3 \times 10^{-10}$, $K_A = 1 \times 10^{-6}$, and $K_l = 4 \times 10^{-6}$. Substitution of these into Eq. 8 gives

$$C^{s}_{B^+} = \frac{D_{B^+} C^{\circ}_{B^+} + 2D_{P=} \{[(3 \times 10^{-10})/(C^{s2}_{B^+})] - [(4 \times 10^{-12})/(C^{\circ 2}_{H^+})]\} + D_{H^+} C^{\circ}_{H^+}}{D_{D^+} + 0.12 D_{H^+}} \quad \text{(Eq. 9)}$$

In the experiments to be discussed later, $C^{\circ}_{H^+} \sim 0.01$ to 0.1 M. For this range of $C^{\circ}_{H^+}$ it can be shown by consideration of Eqs. 4 and 5 that to within a factor of ten, $C^{s}_{B^+} \sim C^{\circ}_{H^+}$. Therefore, the middle terms in the numerator of Eq. 9 are negligible compared to the first and last terms. Hence for our present purposes, Eq. 10 may be written

$$C^{s}_{B^+} = \frac{D_{B^+} C^{\circ}_{B^+} + D_{H^+} C^{\circ}_{H^+}}{D_{B^+} + 0.12\, D_{H^+}} \quad \text{(Eq. 10)}$$

Now if this expression is combined with Eq. 5 we get

$$G = \frac{D_{B^+}}{s} \left(\frac{D_{H^+} C^{\circ}_{H^+} - 0.12\, D_{H^+} C^{\circ}_{B^+}}{D_{B^+} + 0.12\, D_{H^+}} \right) \quad \text{(Eq. 11)}$$

Benzphetamine Release vs. Time Relation.— We may now relate G to the variation in thickness s with time t by means of the following equation

$$G = A \frac{ds}{dt} - {}^1/_2\, V C^{s}_{B^+} \frac{ds}{dt} \quad \text{(Eq. 12)}$$

where A is the concentration of benzphetamine in the solid benzphetamine pamoate and V is the volume fraction of the liquid (aqueous) phase in the pamoic acid layer. The first term on the right side of Eq. 12 is the amount of benzphetamine leaving[3] the solid benzphetamine pamoate per unit time and unit area, and the second term is the amount of benzphetamine needed to re-establish the steady-state concentration gradient in the region $0 \leq x \leq s$ after the change in s.

Letting

$$\Delta C = A - ({}^1/_2)\, V C^{s}_{B^+} \quad \text{(Eq. 13)}$$

combining Eqs. 11 and 12, and separating variables, we get

$$s\,ds = \frac{D_{B^+}}{\Delta C} \left[\frac{D_{H^+} C^{\circ}_{H^+} - 0.12\, D_{H^+} C^{\circ}_{B^+}}{D_{B^+} + 0.12\, D_{H^+}} \right] dt \quad \text{(Eq. 14)}$$

Integrating from $s = 0, t = 0$ to $s = s, t = t$ this becomes

$$s = \left[\frac{2D_{B^+}}{\Delta C} \left(\frac{D_{H^+} C^{\circ}_{H^+} - 0.12 D_{H^+} C^{\circ}_{B^+}}{D_{B^+} + 0.12\, D_{H^+}} \right) t \right]^{1/2} \quad \text{(Eq. 15)}$$

Then for the amount M released per cm.^{2} of surface we have

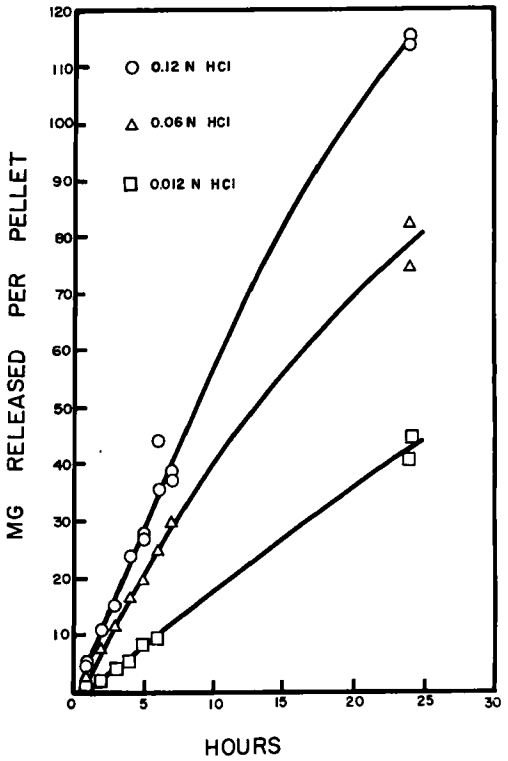

Fig. 2.—Data on the release of benzphetamine from pellet of benzphetamine pamoate in aqueous solutions of hydrochloric acid at 37°.

$$M = S\Delta C = \left[2D_{B^+} \Delta C \left(\frac{D_{H^+} C^{\circ}_{H^+} - 0.12\, D_{H^+} C^{\circ}_{B^+}}{D_{B^+} + 0.12\, D_{H^+}} \right) t \right]^{1/2} \quad \text{(Eq. 16)}$$

In order to make Eq. 16 workable, it is necessary to have estimates of D_{B^+}, D_{H^+}, and ΔC. For the diffusion coefficients we may write

$$D_{B^+} = f D'_{B^+}$$

and (Eq. 17)

$$D_{H^+} = f D'_{H^+}$$

where the D primes are the diffusion coefficients of the ions in pure aqueous phase. Therefore, f represents the effects of volume fraction, particle shapes, and other factors. The value of f may be estimated from approximate theories (6) based on data for other systems. We find that, if $V \sim 0.5$, $f \sim 0.25 \pm$ factor of 50%. The value for D'_{B^+} may be estimated from the Stokes-Einstein law to be about 5×10^{-6} cm.^{2} sec.$^{-1}$ and $D'_{H^+} \sim 3 \times$

[2] These are for 37°. The K_A value was determined at 25° by a potentiometric titration method in various concentrations of aqueous dimethylacetamide solutions and the results were extrapolated to 100% water. The temperature dependence of K_A values in water for carboxylic acids are usually small (5) near room temperatures. Since K_A for salicylic acid is about 1×10^{-3}, our value for pamoic acid is reasonable.

[3] It is assumed that the rate of change in thickness of the pamoic acid layer equals that of the pamoate layer. This is consistent with experimental observations.

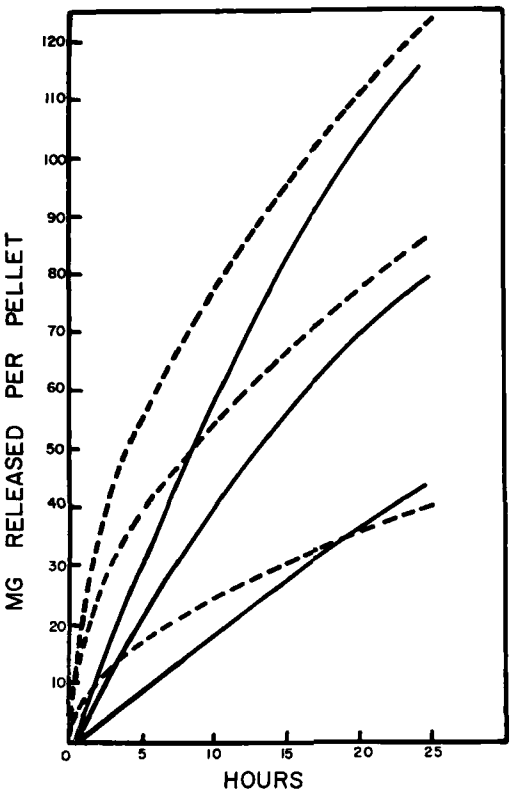

Fig. 3.—Comparison of experimental results (closed curves) with Eq. 19 of theory (dotted curves).

10^{-5} cm.^{2} sec^{-1} from the literature (7). Finally, since it can be easily shown from Eq. 10 and the above estimates of the diffusion coefficients that $C^{\circ}_{D^+} \ll A$ for our experimental conditions, we have $\Delta C \simeq A$. Therefore, $\Delta C \simeq 2.6 \times 10^{-3}$ moles ml.$^{-1}$ since the density of benzphetamine pamoate is 1.12 and the equivalent weight is 433. With these values for D_{B^+}, D_{H^+}, and ΔC, and noting that $C^{\circ}_{H^+} \gg 0.12\ C^{\circ}_{B^+}$ for our experiments, Eq. 16 becomes

$$M \simeq 1.5 \times 10^{-4} (C^{\circ}_{H^+} t)^{1/2} \text{ moles benzphetamine release per cm.}^2 \quad \text{(Eq. 18)}$$

if $C^{\circ}_{H^+}$ is in moles ml.$^{-1}$ and t is in seconds.

EXPERIMENTAL

Because of the large number of parameters in the theory, the values of which are known only approximately, it appeared that an evaluation of the problem on the basis of a single experiment would be difficult. Therefore, it was decided to vary an obviously important factor, the hydrogen ion concentration, to see whether the results of the experiments would fall self-consistently into the framework of the theory.

A series of experiments employing the hanging pellet method (8) at 0.12, 0.06, and 0.012 *N* hydrochloric acid were carried out. The pellets, which were one-half inch diameter and approximately two millimeters thick, were prepared by compressing benzphetamine pamoate in a potassium bromide die. The die was evacuated and a compressional force of 10 tons was applied. The pellets were mounted on aluminum plates by means of wax. Excess wax and any trace of magnesium stearate, which was used to coat the inner surfaces of the die, were removed from the one-half inch diameter surface by careful scrapping with a razor blade. Each pellet was then exposed to 100 ml. of the hydrochloric acid solutions. At a predetermined time a pellet was removed and the amount of drug released by it was determined by U.V. and colorimetric methods. All runs were carried out at 37°.

COMPARISON OF RESULTS WITH THEORY

The data on the release of benzphetamine with time are presented in Fig. 2 along with their best fit curves. Each experimental point represents a different pellet.

These data may be compared with theory in three ways. First, the coefficient, 1.5×10^{-4}, in Eq. 18 may be tested; secondly, the $C^{\circ}_{H^+}$ dependence may be examined; and finally, the time dependence may be tested.

In Fig. 3 the experimental results are compared with the adjusted equation

$$M = 1.25 \times 10^{-4} (C^{\circ}_{H^+} t)^{1/2} \text{ moles cm.}^{-2} \quad \text{(Eq. 19)}$$

which differs from Eq. 18 by only 20% in the coefficient. The surface area of 1.26 cm.^{2} was used with Eq. 19 for the calculation of the theoretical curves.

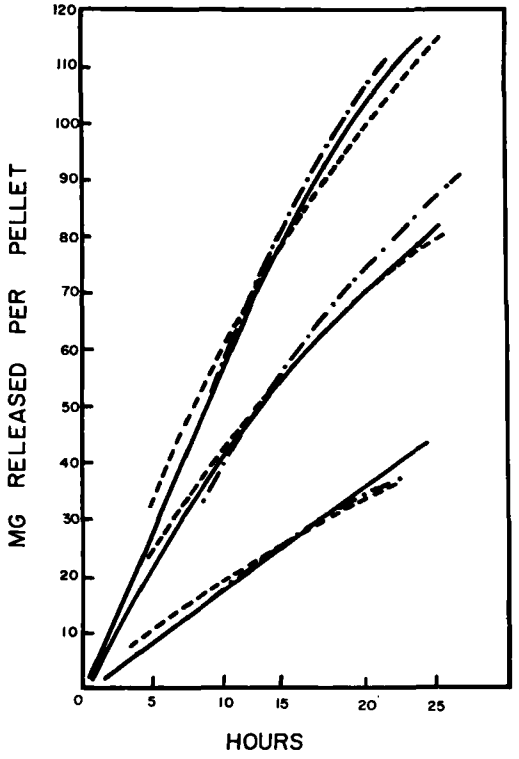

Fig. 4.—Comparison of experimental results (closed curves) with Eq. 20 (–·—·–) and with Eq. 21 (– – – –) of theory.

A close examination of Fig 3 shows that on two of the three tests of the theory with data the theoretical model appears to be in satisfactory agreement with the experiments. Firstly, the experimental $C^{\circ}{}_{H^+}$ dependence correctly fits the square root law[4] at essentially all times. Secondly, within the probable uncertainty of about a factor of two in theory, the coefficient, 1.5×10^{-4}, in Eq. 18, agrees[5] with data at large times for all $C^{\circ}{}_{H^+}$. Thus it appears that the disagreement between experiment and theory exists largely on the third point, the time dependence.

To amplify the idea that it is primarily the time dependence of the model which is at fault, let us consider two modifications of Eq. 18 which give better fit to the data. In Fig. 4 the data are compared with the following two equations

$$M = 1.4 \times 10^{-4} (C^{\circ}{}_{H^+})^{1/2} (t - \tau_1)^{1/2} \quad \text{(Eq. 20)}$$

and

$$M = 1.4 \times 10^{-4} (C^{\circ}{}_{H^+})^{1/2} (t^{1/2} - \tau_2^{1/2}) \quad \text{(Eq. 21)}$$

where we have taken τ_1 = six hours and τ_2 = one hour for the calculations. In these equations the coefficient and $C^{\circ}{}_{H^+}$ dependence have been retained from Eq. 18. Only changes in the time dependence, effective mainly at small t, have been incorporated. The meaning of τ_1, in Eq. 20, is that there is effectively a constant lag time, $\tau_1 \sim$ six hours, before the release process begins according to the theoretical model. The meaning of τ_2 in Eq. 21 is that there is not only a small lag time $\tau_2 \sim$ one hour, but there is effectively a small barrier in series with the pamoic acid barrier itself. This small effective barrier would, of course, be most important at small t values when the pamoic acid layer is thin. Actually, the data in Fig. 2 indicate a small lag time the order of one hour. It is more difficult to account for the barrier in series with the pamoic acid layer. It might be partially accounted for by the liquid diffusion layer and partially by a varying pamoic acid layer structure near the surface, *i.e.*, effectively smaller diffusion coefficients in the layer near the surface.

[4] If the coating phenomenon were absent, a linear law would be expected according to Eq. 11 with constant s.

[5] If the coating phenomenon were absent, rates ten to a hundred times greater would be expected.

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An *in vivo* tracer method for screening drugs which act by altering the normal body metabolism of certain elements is described. These studies involved the detection of natruretic or antinatruretic action of six compounds. Further extensions of the present work are also discussed.

THE RELATIVELY RECENT development of large volume liquid scintillation counters ("Whole Body Counters") (1, 2) has made possible research on the development of new methodology for the qualitative and quantitative evaluation of pharmacologically active substances. *In vivo* assay of gamma ray emitting radioisotopes, as made possible with large volume liquid scintillators, is particularly attractive to the research pharmacologist in drug screening studies. The obvious advantages of this technique are three: (*a*) observations may be made on the intact animal; (*b*) serial observations may be made on the same animal over extended periods of time; and (*c*) it should be possible to reduce the size of experimental groups of animals. This technique has not been exploited to date.

One such counter is the Purdue University Small Animal Counter (PUSAC). The specifications and operating characteristics of this counter are fully described in the literature (2). The PUSAC and similar counters now being commercially produced are of such size that mice, rats, or guinea pigs may be used as experimental animals.

Large volume scintillation counters should

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The authors express their gratitude to the Merck Sharpe and Dohme Co. for the generous supply of chlorothiazide and hydrochlorothiazide, to G. D. Searle and Co. for spironolactone, and to Ciba Pharmaceutical Co. for amphenone B.
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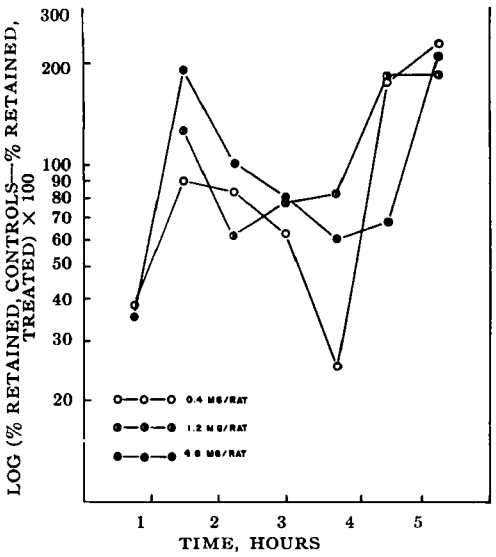

Fig. 1.—Effect of spironolactone on sodium retention in the rat.

provide a sensitive assay method for studying the effects of physiologically active substances on body pool, turnover time, and thus storage and excretion rates of several body ions of interest. However, the following criteria must be met: *A*, The ion must exist in the body in a measurable concentration. *B*, It must have been assigned a specific biochemical role, beneficial or otherwise. *C*, The concentration of the ion must be modified by drug administration or altered in disease conditions, so that a measurable change in concentration reflects the action of the drug or the development of a diseased state. *D*, The ion must have a suitable gamma emitting isotope.

Many ions existing in the body, such as sodium, potassium, calcium, and iodine meet all of the above criteria. Therefore, studies involving drugs or diseased states which alter the normal metabolism of these elements should be possible by the procedures discussed.

Classifications of the various possible studies concerning ions which meet the above criteria are: *A*, the role of the ion in normal physiology, with special reference to body pool, storage time, excretion patterns, and absorption studies; *B*, the effect of drugs in modifying normal body concentrations of the ion and interpretation of results in terms of drug-type and drug-dose, *i.e.*, screening methods; *C*, studies of normal ion concentrations as influenced by disease states. Relationship between body levels of specific metals and type and severity of disease, *i.e.*, diagnostic techniques; *D*, relating to items two and three above, studies of the effect of drug therapy on the diagnostic criteria and indications developed by whole body counting procedures, *i.e.*, evaluation of specific therapeutic procedures as opposed to screening techniques; and *E*, Industrial toxicology.

This study is an example of one of the many possible applications of large volume liquid scintillation counters to pharmacological studies.

The effects of various pharmacologic compounds, mainly diuretic agents, on sodium metabolism in the rat using sodium-22 were studied. This isotope emits a gamma ray of 1.30 Mev., thus making it possible to detect and quantitatively measure the amount of this isotope using external liquid scintillation detectors.

The most common method of studying the diuretic or natruretic activity of pharmacologically active compounds is to collect urine specimens and determine the volumes and/or electrolyte content. The electrolyte content is determined in most cases by flame photometry (3–5); however, this value is not always precise due to interference of trace ions present in the urine.

Since all diuretic agents affect sodium reabsorption in the kidney in some manner, a measure of the amount of sodium retained and by difference the amount excreted should give an indication of the pharmacological activity of the

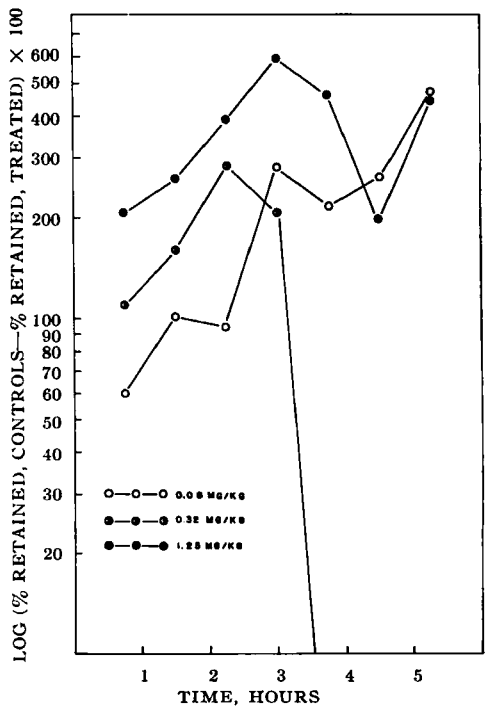

Fig. 2.—Effect of hydrochlorothiazide on sodium retention in the rat.

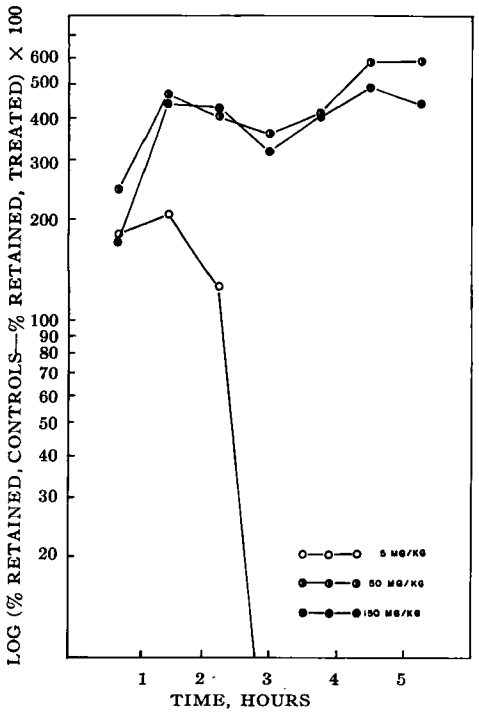

Fig. 3.—Effect of acetazolamide on sodium retention in the rat.

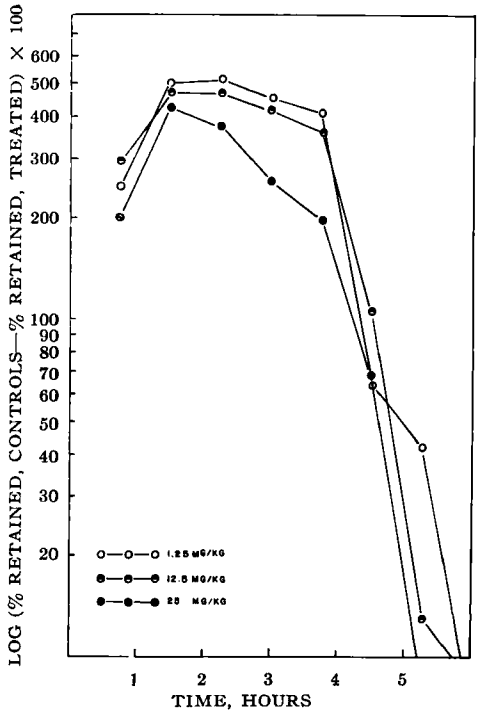

Fig. 4.—Effect of chlorothiazide on sodium retention in the rat.

drug. Wiebelhaus, *et al.* (6), found that if animals used in testing diuretics were first hydrated with 0.9% sodium chloride solution, the resulting values obtained for the natruretic activity of various diuretic compounds also were a good indication of their diuretic activity. This finding has also been reported elsewhere in the literature (7, 8).

In this study rats were hydrated with isotonic sodium chloride solution containing sodium-22. Known diuretic agents were administered and the whole body retention of sodium-22 measured, at several time intervals, as an indicator of natruretic efficacy. In other experiments urine was collected in addition to sodium-22 measurements on the whole animal to provide a correlation between natruretic and diuretic effect.

METHODS

Female Sprague-Dawley rats weighing between 180 and 220 Gm. were used throughout this study unless otherwise noted. The rats received treatment prior to test as indicated.

Desoxycorticosterone Blocking Effect of Spironolactone.[1]—This study dealt with the detection of the ability of spironolactone to relieve desoxycorticosterone acetate (DCA) induced sodium retention in the adrenalectomized rat. Four groups of six animals were adrenalectomized and maintained as described by Kagawa and Brown (3).

At the 24th hour after adrenalectomy, DCA in corn oil (12 mcg. in 0.05 ml.) was injected subcutaneously in the shoulder region of the animals. All animals were then hydrated by injecting subcutaneously 2.5 ml. of 0.9% sodium chloride solution, containing approx. 0.05 μc. of sodium-22, at divided sites in the shoulder region.

Three hours later, at the 27th hour after adrenalectomy, the rats were again injected with DCA as described above. At this time the control group received 0.5 ml. of corn oil subcutaneously, and group I received 0.4 mg.; group II, 1.2 mg.; and group III, 4.8 mg. of spironolactone in 0.5 ml. of corn oil.

The animals were then studied for a period of 5¼ hours by periodically determining the percentage of the original whole body sodium-22 activity remaining by *in vivo* radioassay.

Natruretic Activity of Four Diuretic Agents.—The natruretic activity of four diuretic compounds at three dose levels, chlorothiazide[2] (1.25, 12.5, and 25 mg./Kg.), hydrochlorothiazide[3] (0.08, 0.32, and 1.25 mg./Kg.), acetazolamide[4] (5, 50, and 150 mg./Kg.), and meralluride[5] (0.7, 3.5, and 7.0 mg. Hg/Kg.), was studied. Four groups of six

[1] 3-(3-oxo-7α-Acetylthio-17β-hydroxy-4-androsten-17α-yl) propionic acid γ-lactone. Marketed as Aldactone by G. D. Searle and Co.

[2] 6-Chloro-7-sulfamoyl-2H-2,3,4-benzothiadiazine 1,1-dioxide. Marketed as Diuril by Merck Sharpe and Dohme Co.

[3] 6-Chloro-3,4-dihydro-7-sulfamoyl-2H-1,2,4-benzothiadiazine 1,1-dioxide. Marketed as Hydrodiuril by Merck Sharpe and Dohme Co.

[4] 5-Acetamido-1,3,4-thiadiazole-2-sulfonamide. Marketed as Diamox by Lederle Laboratories.

[5] N[[3-(1,2,3,4,5,6-Hexahydro-1,3-dimethyl-2,6-dioxopurin-7-ylmercuri)-2-methoxypropyl]carbamoyl] succinamic acid. Marketed as Mercuhydrin by Lakeside Laboratories.

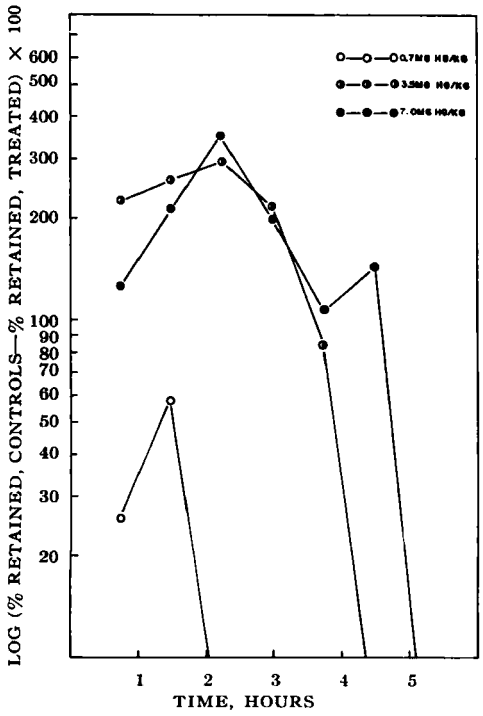

Fig. 5.—Effect of meralluride on sodium retention in the rat.

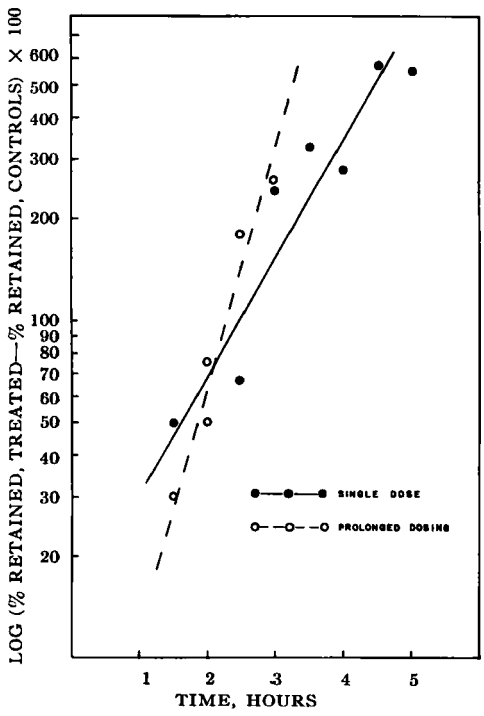

Fig. 6.—Effect of amphenone-B on sodium retention in the rat.

animals were used for each compound. One group was kept as a control in each case.

Hydration consisted of intraperitoneal injection of 25 ml./Kg. of 0.9% sodium chloride solution containing 0.05 μc. of sodium-22. The animals then received an intraperitoneal injection of each compound and control immediately after hydration. The amount of radioactivity retained was then determined by periodically determining the total body radioactivity at 45-minute intervals over 5¼ hours.

All meralluride injections were intramuscular.

Effect of Amphenone B[6] on Sodium Metabolism.—Vogt (9) noted that amphenone B would either increase or decrease sodium excretion in rats dependent upon whether the animal received a single dose of the compound or prolonged doses over a period of 2–17 days. Since the activity of this compound is evidenced by a change in sodium metabolism brought about by a change in aldosterone biosynthesis, it was subjected to examination by this technique.

In the single dose study, rats were injected intraperitoneally with 200 mg./Kg. of amphenone B in distilled water following hydration; the controls received an equal volume of distilled water. In the case of prolonged doses, the animals received 200 mg./Kg. of the compound in distilled water daily for 5 days. Controls again received an equal volume of distilled water at the same time. Hydration was undertaken just prior to the injection of the compound on the fifth day of dosing. Sodium retention was determined as previously described.

[6] 3,3-Bis(*p*-aminophenyl) butane; Ciba Pharmaceutical Products, Inc.

Ability of Diuretic Agents to Concentrate Sodium in the Urine.—Since there is reason to believe that diuretic activity and natruretic activity are related, all conditions in the previous experiments (with the exception of the amphenone B experiment) were duplicated; the animals were placed in metabolism cages and urine samples collected. The radioactivity contained in the samples was determined by placing the urine in a 20-ml. glass bottle and then placing in the PUSAC. The percentage of the original whole body radioactivity that was contained in the sample was determined.

RESULTS AND DISCUSSION

It was possible to detect effects on sodium metabolism elicited by each of the drugs used. Both natruretic responses and inhibition of sodium excretion were readily observed.

The results obtained in the experiments involving the DCA-blocking effect of spironolactone indicate a positive response at each dose administered (Fig. 1.)[7] These results suggest that the drug has a rapid onset and a duration of action of over 5 hours as well under the conditions of this experiment. The changes in slope are possibly explained by the mode of administration of DCA, since two oily subcutaneous injections were made 3 hours apart.

[7] In Fig. 1 and in all figures showing natruretic activity, a semilogarithmic plot was obtained by plotting the difference of the per cent of sodium retained by the controls, minus the per cent retained by the treated animals, multiplied by 100, along the *y*-axis and time along the *x*-axis. Where sodium retention was indicated, the difference of the per cent retained by the treated animals, minus the per cent retained by the controls, multiplied by 100, was logarithmically plotted on the *y*-axis.

TABLE I.—STUDIES OF SODIUM CONCENTRATION IN THE URINE[a]

Drug	Av. Urine Vol.	% of Orig. Act.[b]	DI, ml. %[c]
Acetazolamide			
Control	7.2	4.7	33.7
Treated	8.5	8.8	75.1
Chlorothiazide			
Control	8.9	7.2	64.4
Treated	6.4	12.2	77.8
Hydrochlorothiazide			
Control	6.3	6.6	41.4
Treated	10.3	8.8	91.8
Meralluride (10–20 hours)			
[d]Control	5.7	4.1	23.3
[d]Treated	12.3	2.7	37.1
Spironolactone			
Control	5.1	1.2	6.0
Treated	7.3	2.0	14.5

[a] Five-hour samples unless otherwise stated. Doses: acetazolamide (50 mg./Kg. BW), chlorothiazide (12.5 mg./Kg. BW), hydrochlorothiazide (2.5 mg./Kg. BW), meralluride (3.5 mg. Hg/Kg. BW), spironolactone (1.2 mg./rat). [b] $\frac{\text{Activity in urine specimen}}{\text{Activity in whole animal at time } 0} \times 100$. [c] Urine volume × av. per cent of original activity. [d] Data from six animals, all others from three animals.

This could allow for a fluctuation in the blood level of DCA.

The results obtained from the studies involving the four known diuretic agents are shown in Figs. 2–5. In each case it was possible to detect the natruretic activity of the drug under study in the chosen test period. These compounds represent three physiologically different natruretic actions, suggesting the feasibility of this procedure for screening diuretic agents. The results also indicate a possibility that more elaborate experimental setups, primarily a more varied dose schedule, might give quantitative as well as qualitative indications regarding the efficacy of a particular drug. This was evidenced by the fact that the results obtained here closely correlate with the known action of the drugs in respect to onset and duration of action.

Amphenone B was shown to inhibit sodium excretion as a single dose or after prolonged doses. These results were reproducible under the conditions of these experiments, although they are not in complete agreement with earlier experiments reported in the literature (10).

Ability of Diuretic Agents to Concentrate Sodium in the Urine.—Results in Table I indicate that neither urine volume measurements alone nor sodium-22 excretion alone gave consistent indications of the activity of the known diuretic compounds in every case. The explanation for this may be that the experimental groups in this particular study were small and that the periods of observation were short. However, the DI (Diuretic Index) did give definite indications of drug action in all cases studied here. (See Table I.) (DI = Urine Volume × Average % of Original Whole Body Radioactivity in Urine.) The percentage of original activity was calculated by dividing the net amount of activity contained in the urine sample by the net amount of activity contained in the corresponding animal at time *0*, and then converting to per cent. This value was calculated for each animal, and the average of the values was determined for each test compound.

It appears from these results that an enhancing or inhibiting effect on sodium excretion by compounds known to affect sodium metabolism could be determined in a simple 5-hour experiment.

SUMMARY

Procedures have been described for detecting drug effects on sodium metabolism in rats over a period of 5 hours by whole body radioassay techniques in an effort to evaluate the feasibility of *in vivo* tracer techniques in drug screening studies. These procedures have the following advantages: short experiment times are possible; it is not necessary to collect urine samples; fewer test animals are required than in diuretic screening procedures presently in use; and it is not necessary to sacrifice the animal, thereby allowing for serial study of drug action on the same animal.

The procedures described here involve studies of sodium metabolism only; however, whole body liquid scintillation counting should be an effective tool in investigations of pharmacologically active substances, especially in cases where the drug in question exerts its major effect on a body constituent which is available in a labeled form. Compartmental analyses of the excretion curves of many metabolically active ions (Na, K, Ca, Fe, I) may be expected to serve qualitatively and quantitatively as sensitive indicators of drug action.

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Reduction Studies on 3,4-Dihydroxyphenylglyoxylohydroxamyl Chlorides and Related Compounds

By JOSEPH P. LAROCCA and GEORGE R. McCLURE†

The preparation of four new phenylglyoxylohydroxamamides is described; however, only two of these products are fully characterized. The reduction of 3,4-dihydroxyphenylglyoxylohydroxamyl chloride and N-α-methylbenzylphenylglyoxylohydroxamamide with platinum-palladium catalyst under low pressure has been studied and conclusions drawn from chemical, physical, and analytical data.

In 1951, LaRocca, *et al.* (1), were able to reduce successfully phenylglyoxylohydroxamyl chloride to phenethanolamine by utilizing a mixed palladium-platinum catalyst. In addition, they prepared and reduced several arylglyoxylohydroxamamides. Efforts to prepare norepinephrine, either by reduction of the corresponding 3,4-dihydroxyphenylgloxylohydroxamyl chloride or by 3,4-dihydroxyphenylglyoxylohydroxamamide were unsuccessful, since the reduction products could not be identified. The purpose of this investigation is to study these reductions in an attempt to clarify the reaction.

EXPERIMENTAL

Low pressure (approx. 4 Atm.) and room temperature were used for all reductions. These were carried out in a Parr series 3910 hydrogenation apparatus. The platinum oxide used for the preparation of the mixed catalyst itself was prepared from platinum chloride by fusion with potassium nitrate as described in Reference 2. The mixed catalyst used in all reductions was composed of: 10.00 Gm. activated charcoal, 1.00 Gm. palladium chloride, 0.15 Gm. platinum oxide.

The catalyst was prepared by suspending these materials in 100 ml. of 1 *N* sodium acetate solution and shaking under 1 atmosphere of hydrogen until hydrogen uptake ceased. The catalyst was then filtered off and washed with several portions of distilled water, followed by ethyl alcohol. Attempts to dry the catalyst by suction usually resulted in spontaneous ignition of the catalyst so it was used while still damp.

Reduction of 3,4-Dihydroxyphenylglyoxylohydroxamyl Chloride.—The reduction of 4.27 Gm. (0.02 mole) of 3,4-dihydroxyphenylglyoxylohydroxamyl chloride was carried out by shaking (under 4 Atm. of hydrogen) an acidified hydroalcoholic solution (95 ml. of 95% ethanol and 5 ml. of concentrated hydrochloric acid) of the compound with the mixed catalyst described above.

The first mole of hydrogen was absorbed in 2 minutes, the second in 20 minutes, the third in 3 hours, and the fourth in 6 hours. From the reduction mixture, 2.9 Gm. (77.5% yield based on 3,4-dihydroxyphenethylamine hydrochloride) of a colorless solid was obtained. Repeated recrystallizations from hot absolute alcohol and benzene gave colorless plates which melted at 243°dec. The melting point of norephinephrine hydrochloride is 141°. This reduction was repeated 10 times with similar results. The reduction product was soluble in water, slightly soluble in alcohol, and insoluble in benzene and ether. It gave an emerald green color with ferric chloride solution, indicating an *o*-dihydroxyphenyl nucleus and (with aqueous silver nitrate) a precipitate of silver chloride which is quickly reduced to free silver. It is very susceptible to oxidation in alkaline solution, first giving a rose colored solution which darkens on standing.

Anal.—Found: C, 50.99 and 51.00; H, 6.45 and 6.45; Cl, 18.78 and 18.78; N, 7.15 and 7.24.[1] The best emprical formula obtained from this information is $C_8H_{12}ClNO_2$.

A quantitative determination of acetylatable groups by the method described by Siggia (3) indicated the presence of two such groups. However, the development of a rose color in alkaline solution indicated that at least one functional group had not been acetylated.

The infrared spectrum of the reduction product was determined on a 500-mg. potassium bromide disk containing 50 mg. of sample. This spectrum was determined on a Perkin Elmer model 112, double pass, single beam spectrophotometer. The interpretation of the spectrum was made from an energy transmission record. The presence of a NH_3^+ group was indicated by bands at 3.29, 6.18, and 6.78 μ; a hydroxy (or secondary amine) group gave a strong band at 3.02 μ. Bands at 6.24, 6.32, 6.56, 7.90, 9.05, 9.26, 10.38, 11.43, and 12.27 μ confirm the presence of a 1, 2, 4-trisubstituted benzene ring. Strong bands at 7.78 and either 7.46 or 7.58 μ show the presence of one or more phenolic hydroxyl groups.[2] The above data coupled with the ease of reducibility of hydroxyl groups on carbon atoms adjacent to the benzene ring seem to establish fairly conclusively that the reduction product is 3,4-dihydroxyphenethylamine hydrochloride. This is an agreement with the best empirical formula as determined from the analytical data. The injection of 1.0 ml. of a 1/200 *N* solution of the reduction product into a dog under pentobarbital anesthesia

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[1] All microanalyses by Oakwold Laboratories, Alexandria, Va.

[2] The infrared spectrum of this compound was determined and interpreted by Dr. George E. Philbrook University of Georgia Chemistry Department, Athens.

TABLE I.—SUMMARY OF ARYLGLYOXYLOHYDROXAMAMIDES

$C_6H_5 \cdot CO \cdot C(NOH) \cdot NR'R''$ R′	R″	M. p.[a]	Description	Yield, %	Nitrogen—Calcd.	Nitrogen—Found
Cyclohexyl	H	...	Brown oil	98.3	...	...
Ethyl	Ethyl	...	Yellow oil	119.0	...	...
						10.78
p-Methylphenyl	H	154°	Tan needles	74.0	11.01	11.03
						9.59
α-Methylbenzyl	H	128°	Yellow plates	88.0	10.44	9.67

[a] All melting points are uncorrected.

produced a pressor response similar to that obtained by Barger and Dale (4) with an equal dose of 3,4-dihydroxyphenethylamine hydrochloride. The melting points of the reduction product and 3,4-dihydroxyphenethylamine hydrochloride are in close agreement: literature value for 3,4-dihydroxyphenethylamine hydrochloride 240–241° dec. (5), reduction product 243° dec. The picrate of 3,4-dihydroxyphenethylamine is reported to melt at 189° (5); the picrate of the reduction product melts at 190–193°. In all instances reduction of 3,4-dihydroxyphenylglyoxylohydroxamyl chloride gave a small amount of a yellow oil in addition to the colorless crystalline 3,4-dihydroxyphenethylamine hydrochloride. This oil was found to be extremely hygroscopic, and it underwent color change in alkaline solution. Upon addition of a base, the solution became cherry red in color and changed to yellow when an acid was added. These properties were not possessed by either 3,4-dihydroxyphenylglyoxylohydroxamyl chloride or the 3,4-dihydroxyphenethylamine hydrochloride produced in the reduction reaction.

Preparation of Arylglyoxylohydroxamamides.—The arylglyoxylohydroxamamides were prepared by essentially the same method reported by LaRocca, *et al.* (1). As was expected, the aromatic amides were found to be crystalline solids, and the aliphatic amides to be resinous solids or oils. Attempts to prepare the hydrochlorides of the aliphatic amides failed to give crystalline solids. (See Table I.)

Reduction of N-α-methylbenzylphenylglyoxylohydroxamamide.—In a reduction solution identical with that previously described, 5.26 Gm. (0.02 mole) of N-α-methylbenzylphenylglyoxylohydroxamamide was shaken under 4 Atm. of hydrogen for 40 hours. During this time 3.5 moles of hydrogen were taken up. Removal of the catalyst and evaporation of the solvent under reduced pressure gave 4.5 Gm. of a yellowish white solid which melted at 154°. Repeated recrystallizations gave colorless plates which melted at 169–171.°

Anal.—Found: Cl, 11.73 and 11.56; N, 9.18 and 9.17. The best empirical formula which can be assigned from the analytical data is $C_{16}H_{19}Cl\ N_2O_2$ which indicates the reduction of the ketone group.

An infrared spectrum was determined on a Nujol mul of the compound. This spectrum showed a band at 3.02 μ (which is characteristic of a hydroxyl or secondary amino group), a strong band at 6 μ (indicative of a C=O or C=N), and bands at 6.29, 6.32, and 6.56 μ (indicative of a phenyl group).[3] These data indicate that the oximino alcohol is the product of the reduction. Attempts to reduce this compound further by the above procedure were unsuccessful.

[3] The infrared spectrum of this compound was determined and interpreted by the Department of Research in Chemical Physics, Mellon Institute of Industrial Research, Pittsburgh, Pa.

DISCUSSION AND CONCLUSIONS

The successful reduction of phenyl-, *p*-xynyl-, *p*-methylphenyl-, and probably 3,4-dihydroxyphenyl, glyoxylohydroxamyl chlorides to the corresponding arylethanolamines by the use of hydrogen under high pressure with a platinum-palladium catalyst has been reported (1). Attempts to reduce 3,4-dihydroxyphenylglyoxylohydroxamyl chloride with the same type catalyst under low pressure failed to give the corresponding phenethanolamine. The pharmacological, chemical, analytical, and physical data indicate that the reduction product is 3,4-dihydroxyphenethylamine. The same type data for the reduction product of N-α-methylbenzylphenylglyoxylohydroxamamide strongly indicates the formation of the corresponding oximino alcohol. These data indicate that, in the reduction of 3,4-dihydroxyphenylglyoxylohydroxamyl chloride, the oxygen atom on the carbon adjacent to the aromatic nucleus is removed by the reduction. In the case of phenyl-, *p*-xenyl-, *p*-methyl- phenylglyoxylohyoroxamyl chlorides and N-α-methylbenzylphenylglyoxylohydroxamamide it appears that this oxygen undergoes normal reduction to the corresponding alcoholic group. The most obvious explanation of this phenomena is that certain types of substituents in the aromatic nucleus (at least 3,4-dihydroxy substitution) tend to increase the susceptibility of the oxygen on the α carbon to over-reduction.

Hartung, *et al.* (6), report the reduction of α-isonitrosopropiophenone with palladium-charcoal catalyst proceeds to the amino-ketone first and then to the amino alcohol. All data acquired in this study indicate that, with a platinum-palladium-charcoal catalyst, reduction of the ketone group precedes the reduction of the oximino group. This conclusion is strengthened by the typical oxime color changes in acid and alkaline solution exhibited by the yellow oil by-product obtained from reduction of 3,4-dihydroxyphenylglyoxylohydroxamyl chloride.

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Plate Assays for Griseofulvin in Pharmaceutical Preparations and Body Fluids

By EVERETT W. KNOLL, FRANCES W. BOWMAN, and AMIEL KIRSHBAUM

A microbiological assay for griseofulvin in pharmaceutical preparations using either cylinders or paper disks is described. A modification to assay griseofulvin concentrations in body fluids is also presented. Both assays offer more precision in assaying griseofulvin than existing microbiological methods.

GRISEOFULVIN, an orally effective antifungal agent, produced by *Penicillium griseofulvin* and other species of *Penicillium*, was first isolated in pure form in 1946 (1). Since that time it has been assayed by spectrophotometric (2), polarimetric (3), isotope dilution (3), and microbiological methods (1). In the latter method, solutions containing griseofulvin are serially diluted in nutrient broth which is later seeded with *Botrytis alli*. The greatest dilution causing distortion or curling of the hyphae of *Bortrytis alli* is recorded and used to estimate the potency of the solution. However, this procedure lacks precision and reproducibility, as do most methods employing observation of morphogenetic responses in microorganisms.

A microbiological assay was developed for griseofulvin in pharmaceutical preparations and in serum. The method is a modification of the cylinder-plate agar diffusion assay for penicillin (4). The response is a well-defined zone of mold inhibition which can be accurately measured by its diameter.

MATERIALS AND METHODS

Medium I.—Dextrose 40.0 Gm., peptone 10.0 Gm., and distilled water sufficient to make 1000 ml. The pH should be 5.65 ±0.05 after sterilization.

Medium II.—Dextrose 40.0 Gm., peptone 10.0 Gm., agar 15.0 Gm., chloramphenicol U.S.P. 0.05 Gm. (activity), and distilled water sufficient to make 1000 ml. The pH should be 5.65 ±0.05 after sterilization.

Medium III.—Dextrose 40.0 Gm., peptone 10.0 Gm., agar 15.0 Gm., chloramphenicol U.S.P. 0.05 Gm. (activity), and distilled water sufficient to make 1000 ml. The pH should be 5.65 ±0.05 after sterilization. To each 100 ml. of this agar at approximately 50° aseptically add 2 ml. of a sterile solution of cycloheximide. The solution contains 10 mg./ml. and is prepared by dissolving cycloheximide in distilled water. The solution is sterilized by filtering through a membrane filter having a porosity of 0.22 μ.

Preparation of Spore Suspension.—Grow *Microsporum gypseum* (ATCC 14683) for 3 weeks at 25° in four 3-L. Erlenmeyer flasks, each containing 200 ml. of *Medium I*. Remove the floating mat from the flask with a sterile wire loop and place in a sterile blending jar. Aseptically add 200 ml. of sterile distilled water and blend for approximately 30 seconds, allow to settle, then decant about 150 ml. of the supernatant into 100-ml. sterile, capped centrifuge tubes. Add the amount of distilled water equal to the amount removed each time and repeat this process four to five times to assure maximum recovery of spores from the mycelial mat. Centrifuge the tubes containing the spores at 4000 r.p.m. for 15 minutes and discard the supernatant. Wash the residual spores from all tubes, using a minimum of sterile distilled water (2–3 ml. per tube) and pool the washings into a sterile flask containing a few sterile glass beads. (This procedure usually yields about 25–35 ml. of spore suspension.) The spore suspension is stable for at least 2 months at 5°. Prepare test plates using a 6-ml. base layer of *Medium II* covered with a 4-ml. seed layer of *Medium III* inoculated with varying volumes of spore suspension, *e.g.*, 1 or 2%. Determine the proper per cent of inoculum to be used by observation of the largest and clearest zones of inhibition given by 5 mcg./ml. of griseofulvin on the test plate.

Add 6 ml. of *Medium II* to a 20 × 100-mm. Petri dish and allow to harden for use as a base layer. Cover the Petri dishes with sterile porcelain lids glazed on the outside. Add the proper amount (as previously determined) of the spore suspension to each 100 ml. of *Medium III* which has been cooled to approximately 50°. Mix the culture and agar thoroughly and add 4 ml. to each plate containing the uninoculated base agar. Tilt each plate back and forth to spread the inoculated agar evenly over the surface. When the agar has hardened place six cylinders on the surface so that they are at approximately 60°-intervals on a 2.8 cm. radius. Use stainless steel cylinders that have an outside diameter of 8 ±0.1 mm., an inside diameter of 6 ±0.1 mm., and a 10 ±0.1-mm. length.

Assay of Pharmaceutical Preparations.—Store the griseofulvin working standard at room temperature under desiccation. Dissolve about 50 mg. in sufficient N,N-dimethylformamide (DMF) to give a concentration of 1000 mcg./ml. This solution may be kept for 3 months under refrigeration. Prepare the standard curve by diluting the 1000 mcg./ml. griseofulvin solution to 64, 80, 100, 125, and 156 mcg./ml. in DMF. Then further dilute one part of each of these solutions with 19 parts of 0.1 *M* phosphate buffer pH 8.0 to give final solutions of 3.2, 4.0, 5.0, 6.25, and 7.8 mcg./ml.

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Use three plates for the determination of each point on the curve, except the 5.0 mcg./ml. solution, a total of 12 plates. On each of three plates fill three cylinders with the 5.0 mcg./ml. solution and the other three cylinders with the concentration of the standard curve under test. There will be thirty-six 5.0 mcg./ml. determinations and nine determinations for each of the other points on the curve. Incubate the plates for at least 48 hours at 30°, and measure the diameters of the circles of inhibition. (Plates may be incubated longer if zones are indistinct.) Average the 36 readings of the 5.0 mcg./ml. concentration and the readings of the other concentrations for each set of three plates. The average of the 36 readings of the 5.0 mcg./ml. concentration is the correction point for the curve. Correct the average value obtained for each point to the figure it would be if the 5.0 mcg./ml. reading for that set of three plates were the same as the correction point. Thus, if in correcting the 4.0 mcg./ml. concentration, the average of the 36 readings of the 5.0 mcg./ml. concentration is 16.6 mm., and the average of the 5.0 mcg./ml. concentration of this set of three plates is 16.4 mm., the correction is plus 0.2 mm. If the average reading of the 4.0 mcg./ml. concentration of these same three plates is 14.8, the corrected value is then 15.0 mm. Plot these corrected values, including the average of the 5.0 mcg./ml. concentration on semilogarithmic paper, placing the griseofulvin concentrations on the logarithmic scale and the diameters of the zones on the arithmetic scale. Draw the line of best fit by inspection or by calculation as described by Deutschberger and Kirshbaum (5).

Dissolve an appropriate aliquot of the griseofulvin powder or suspension in DMF to give a convenient stock solution. Further dilute with DMF to give a concentration of 100 mcg./ml. Then further dilute one part of this with 19 parts of 0.1 *M* phosphate buffer pH 8.0 to give a final estimated concentration of 5.0 mcg./ml. of griseofulvin. For the assay of tablets, pool a representative number of tablets (usually five), blend them in 250 ml. of DMF, and follow the dilution procedure described for powders or suspensions.

Use three plates for each unknown sample. Fill three cylinders on each plate with the standard 5.0 mcg./ml. solution and three cylinders with the sample solution to be tested, alternating standard and sample. Incubate the plates for at least 48 hours at 30° and measure the diameter of each circle of inhibition. To calculate the potency of the sample, average the zone readings of the standard and the zone readings of the sample on each set of plates. If the sample gives larger average zone size than the standard, add the difference between them to the 5.0 mcg./ml. zone on the standard curve. If the average sample value is lower than the standard value, subtract the difference between them from the 5.0 mcg./ml. value on the curve. Read from the standard curve the concentration corresponding to the corrected zone size.

Assay of Potency of Pharmaceutical Preparations Using Paper Disks in Lieu of Cylinders.—In the method described above, paper disks may be substituted for the cylinders if preferred by making the following changes. Use round, blank disks having a diameter of 1/4 in. made of clear-white filter paper. Schleicher and Scheull No. 740-E paper or comparable grade may be used. Place blank disks on aluminum or stainless steel wire mesh which is supported in a manner to allow circulation of air above and below the disks. Prepare the solutions for the standard disks by further diluting the standard stock solution of griseofulvin in DMF to obtain solutions containing the following concentrations: 50, 62.5, 75, 95, and 120 mcg./ml. Pipet 0.02 ml. of each stock solution to three replicate disks, and put in vacuum desiccator for 2–3 hours until perfectly dry. The final concentration of griseofulvin per disk is 1.0, 1.25, 1.5, 1.9, and 2.4 mcg. Disks may be used for assay purposes that have been stored for up to 2 weeks under refrigeration. However, griseofulvin disks stored in stoppered test tubes containing a desiccant showed no loss of potency after 6 months.

Using disks prepared from the five different concentrations of standard described above, proceed as directed for establishing the standard curve by the cylinder-plate method, but in lieu of filling the cylinders, place the disks on the plates with forceps and tap gently to ensure an even seal. Use three plates (prepared as described for cylinder-plate method) for the determination of each point on the curve. On each of three plates, place three 1.5 mcg. disks and three disks containing one of the antibiotic concentrations of the standard curve. There will be thirty-six 1.5 mcg. determinations and nine determinations for each of the other points on the curve.

Prepare the stock solution of the sample as described for the cylinder plate assay. Then further dilute with DMF to a concentration of 75 mcg./ml. Prepare disks from this solution to give a final estimated concentration of 1.5 mcg./disk as described for the standard curves. Incubate the plates for at least 48 hours at 30°. Accurately measure the zones of inhibition with calipers, by projection on a calibrated screen, or by any other suitable method. Plot the standard curve and calculate the potency of the sample as described for the cylinder plate assay.

Assay of Griseofulvin in Body Fluids.—Prepare the suspension, standard curve, and plates as described for assay of griseofulvin in pharmaceutical preparations by the cylinder-plate method except modify preparation of the plates, and make 1 +19 dilutions for the standard curve using the appropriate body fluid. For example, if the body fluid to be assayed for griseofulvin content is serum, use antibiotic-free normal human serum instead of buffer for the final dilutions of the standard. Add the griseofulvin in DMF directly to the serum and shake immediately to distribute the antibiotic evenly through the serum. Prepare stock griseofulvin solutions in DMF to contain 90, 60, 40, 26, and 18 mcg./ml., and add 0.5 ml. of each solution to 9.5 ml. of serum to obtain final concentration of 4.5, 3.0, 2.0, 1.3, and 0.9 mcg./ml.

To prepare the plates, use heavy flat-bottomed (20 × 100 mm.) glass Petri dishes covered with sterile porcelain lids glazed on the outside. Add 5 ml. of *Medium II* seeded with 1% of the spore suspension previously described and allow to harden. Use one plate for each unknown tested. Test serum samples undiluted unless a concentration of greater than 4.5 mcg./ml. is expected. If so, dilute with serum to an estimated concentration of 2.0

mcg./ml. Add the 2.0 mcg./ml. standard solution to alternate cylinders on each plate and the sample to the remaining cylinders. Incubate the plates for at least 48 hours at 30°, read the zones of inhibition, and calculate the concentration of griseofulvin as described for the cylinder plate assay. The lowest concentration of griseofulvin that this method can detect in body fluids is 0.9 mcg./ml.

EXPERIMENTAL

Griseofulvin bulk material, tablets, and suspensions assayed by this cylinder plate or disk method gave results comparable to those obtained using a spectrophotometric method (2) as shown in Table I. Using the described method, standard curves were run using a commercial bulk sample of griseofulvin as a house standard. Four replicate assays of a bulk sample from a different manufacturer were performed on each of three consecutive days. From the results obtained the 95% confidence limits of a single assay of the method described for pharmaceutical preparations were calculated and shown to be ±10.9%. This range can be reduced of course by performing replicate assays. Different commercial preparations were assayed by this procedure and the spectrophotometric method (2). The results summarized in Table I show good agreement.

TABLE I.—GRISEOFULVIN PHARMACEUTICAL PREPARATIONS ASSAYED BY TWO METHODS

Product	Label Potency	Spectrophotometric	Microbial
Bulk	1,000 mcg./mg.	1000	1000
		978	1008
Tablets	250 mg./tablet	250	255
Oral suspension	250 mg./5 ml.	250	250

Experimental studies on the assay in serum were limited because of lack of clinical facilities. However, one male receiving therapy of griseofulvin (250 mg. every 4 hours, total dose 1 Gm./day) for 1 week volunteered to have his serum assayed for griseofulvin. Blood sample was taken 2 hours after a 250 mg. dose of griseofulvin. His serum assayed 0.9 mcg./ml., whereas the serum from a male volunteer receiving no drug was negative.

REFERENCES

(1) Brian, P. W., Curtis, P. J., and Hemming, H. G., *Trans. Brit. Mycol. Soc.*, **29**, 173(1946).
(2) Ashton, G. C., and Brown, A. P., *Analyst*, **81**, 221 (1956).
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Basic 1,3-Dioxolanes

By A. R. PATEL and J. F. ONETO

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Subsequently, extensive investigations on the autonomic pharmacodynamics of basic 1,3-dioxolanes and their quaternary derivatives have been reported (2–8). Recently, Hardie and co-workers (9) have reported on the local anesthetic and spasmolytic properties of a series of 4-(2-piperidyl)-1,3-dioxolanes.

The present work was undertaken to prepare basic dioxolanes with additional structural variations for pharmacologic evaluation. The products represent essentially an extension of the Blicke series (5) of 2-aminomethyl and 4-aminomethyl substituted 1,3-dioxolanes.

The intermediate halodioxolanes (Tables I and II) required for the synthesis of the aminodioxolanes were prepared by two general methods: (*a*) condensation of an α-haloketone with a 1,2-glycol or with glycerol-α-monochlorohydrin in the presence of *p*-toluenesulfonic acid according to the procedure of Salmi (10); (*b*) condensation of a ketone with epibromohydrin in the presence of stannic chloride according to the procedure of Bersin and Willfang (11, 12).

The infrared absorption spectra of a number of the halodioxolanes listed in Table I are shown in Table III. The four bands found in the 990–

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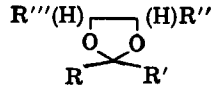
TABLE I.—CONDENSATION PRODUCTS OF α-HALOKETONES WITH 1,2-GLYCOLS[i]



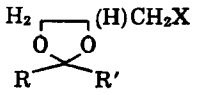
No.	R	R′	R″	R‴	Reflux, Hr.	M.p., °C.	Yield, %	Formula	Analyses, % — Halogen — Calcd.	Found
1[a]	C_6H_5	CH_2Br	H	H	15	59–60	61	$C_{10}H_{11}BrO_2$	32.87	33.01
2	*p*-BrC_6H_4	CH_2Br	H	H	16	82–83	61	$C_{10}H_{10}Br_2O_2$	49.64	49.50
3	*p*-BrC_6H_4	CH_2Br	CH_3	H	9	65–66	48	$C_{11}H_{12}Br_2O_2$	[b]	...
4	*p*-ClC_6H_4	CH_2Br	H	H	22	61 to 62.5	67	$C_{10}H_{10}BrClO_2$	28.79	28.50[c]
5	*p*-ClC_6H_4	CH_2Br	CH_3	H	24	73 to 74.5	50	$C_{11}H_{12}BrClO_2$	[b]	...
6	*p*-ClC_6H_4	CH_2Br	CH_3	CH_3	24	84–89	40	$C_{12}H_{14}BrClO_2$	[b]	...
7[d]	*p*-$CH_3OC_6H_4$	CH_2Br	H	H	12	73.5 to 75	50	$C_{11}H_{13}BrO_3$	29.26	29.46
8	*m*-$NO_2C_6H_4$	CH_2Br	H	H	15	89.5–90.5	62	$C_{10}H_{10}BrNO_4$	27.74	27.82
9	*p*-$NO_2C_6H_4$	CH_2Br	H	H	10	130–133	59	$C_{10}H_{10}BrNO_4$	27.74	27.60
10	*p*-$NO_2C_6H_4$	CH_2Br	CH_3	H	8	126.5 to 128	66	$C_{11}H_{12}BrNO_4$	26.45	26.28
11	*p*-$C_6H_5C_6H_4$	CH_2Br	H	H	15	79.5 to 80	76	$C_{16}H_{15}BrO_2$	25.04	24.92
12	*p*-$C_6H_5C_6H_4$	CH_2Cl	H	H	18	77–78	68	$C_{16}H_{15}ClO_2$	12.91	12.86
13	C_8H_9[e]	CH_2Br	H	H	17	47.5 to 48	65	$C_{12}H_{15}BrO_2$	29.47	29.68
14	C_6H_5	$C(CH_3)_2Br$	H	H	10	74–76	26	$C_{12}H_{15}BrO_2$	29.47	29.24
15	C_6H_5	$C(CH_3)_2Br$	CH_3	H	18	56 to 57.5	22	$C_{13}H_{17}BrO_2$	28.02	27.85
16	C_6H_5	$C(CH_3)_2Br$	CH_3	CH_3	36	91–96	33	$C_{14}H_{19}BrO_2$	26.71	26.62
17	C_6H_5	$C(CH_3)_2Cl$	H	H	7	73.5 to 75	45	$C_{12}H_{15}ClO_2$	15.64	15.71
18	C_6H_5	$C(CH_3)_2Cl$	CH_3	CH_3	24	83.5 to 85	22	$C_{14}H_{19}ClO_2$	13.92	13.82
19[f]	C_6H_5	$CH(Cl)C_6H_5$	H	H	10	74–75	54	$C_{16}H_{15}ClO_2$	12.90	12.65
20	C_6H_5	$C_6H_{10}Br$[g]	H	H	24	119–121	18	$C_{18}H_{19}BrO_2$	25.68	25.48
21	C_6H_5	$C_6H_{10}Cl$[h]	H	H	30	105–106	38	$C_{18}H_{19}ClO_2$	13.29	13.07

[a] Described by Kühn (14). [b] The unanalyzed products were aminated. [c] Analyzed for bromine. [d] Described by Thomae (15). [e] 2,5-Dimethylphenyl. [f] Described by Summerbell and Berger (16). [g] 1-Bromocyclohexyl. [h] 1-Chlorocyclohexyl. All compounds were prepared following the method of Salmi (10). [i] Compounds 2,4, 7, 12, 14, 17, and 19 were recrystallized from methanol, the remainder from ethanol.

1200 cm.$^{-1}$ region are in agreement with the reported observations ascribed by Bergmann and Pinchas (13) as being specific for the C—O—C—O—C grouping. However, Lagrange, and Mastagli (13) have pointed out that many of these bands appear in dioxane derivatives also and that they are not always all present in dioxolanes.

The 4-aminomethyl-1,3-dioxolanes (Table IV) were prepared by heating a mixture of the inter-

TABLE II.—4-HALOMETHYL-1,3-DIOXOLANES



No.	R	R′	X	Reflux, Hr.	B.p., °C./mm.	Yield, %
1	*p*-BrC_6H_4	CH_3	Cl	20	115–116/3	86
2	*p*-ClC_6H_4	CH_3	Cl	15	110–111/3	80
3	*p*-$CH_3OC_6H_4$	CH_3	Cl	24	102–104/4	66
4	*p*-$CH_3C_6H_4$	CH_3	Cl	12	110–112/1	59
5	C_6H_5	C_2H_5	Cl	20	106–109/3	75
6	C_6H_5	*n*-C_3H_7	Cl	24	112–114/3	78
7	C_6H_5	$CH(CH_3)_2$	Cl	24	110–112/3	79
8	C_6H_5	$CH(C_2H_5)_2$	Cl	60	108–112/2	45
9	C_6H_5	$CH(C_2H_5)C_4H_9$	Cl	72	130–132/2	59
10	$C_6H_5CH_2$	CH_3	Cl	24	100–104/3	78
11	*p*-$CH_3C_6H_4$	C_6H_5	Cl	36	[a]	...
12	*p*-ClC_6H_4	C_6H_5	Cl	48	[a]	...
13	*n*-C_9H_{19}	CH_3	Br	[b]	117–118/4	71
14	2-C_4H_3S[c]	CH_3	Br	...	100–102/4	87
15[d]	2-C_4H_3S	C_6H_5	Br	...	155–160/2	74
16	$C_6H_5CH_2$	CH_3	Br	...	110–111/3	73
17[d]	$C_6H_5CH_2$	$C_6H_5CH_2$	Br	...	[a]	...
18[d]	*p*-$CH_3C_6H_4$	*p*-$CH_3C_6H_4$	Br	...	[a]	...

[a] The crude products and unanalyzed distilled products were aminated (Table IV). [b] The bromo derivatives, compounds 13–18, were prepared according to the method of Bersin and Willfang (11, 12) which does not involve refluxing. Compounds 1–12 were prepared by the method of Salmi (10). [c] 2-Thienyl. [d] Described by Blicke and co-workers (5).

TABLE III.—INFRARED SPECTRA OF HALOGENATED 1,3-DIOXOLANE DERIVATIVES[a]

No.	1,3-Dioxolanes	Wavenumber (ν), cm.$^{-1}$ 1234–1205	1183–1156	1064–1023	1013–990
1	2-Bromomethyl-2-phenyl-	1220	1169	1042	1000
2	2-Bromomethyl-2-(*p*-bromophenyl)-	1220	1173	1042	1010
3	2-Bromomethyl-2-(*p*-chlorophenyl)-	1220	1162	1039	1013
4	2-Bromomethyl-2-(*p*-methoxyphenyl)-	1215	1169	1030	997
5	2-Bromomethyl-2-(*p*-nitrophenyl)-	1215	1162	1036	1005
6	2-Bromomethyl-2-(*m*-nitrophenyl)-	1227	1177	1044	1002
7	2-Bromomethyl-2-(*p*-phenylphenyl)-	1212	1156	1030	1000
8	2-Chloromethyl-2-(*p*-phenylphenyl)-	1223	1183	1036	1008
9	2-Bromomethyl-2-(2,5-dimethylphenyl)-	1234	1156	1036	990
10	2-(2-Chloroisopropyl)-2-phenyl-	1234	1180	1023	1010
11	2-(α-Chlorobenzyl)-2-phenyl-	1215	1162	1047	1008
12	2-(1-Chlorocyclohexyl)-2-phenyl-	1205	1169	1064	1005

[a] Measured in potassium bromide disks (concentration 0.4%; disk thickness 0.6 mm.) with a Perkin-Elmer model 21 infrared spectrophotometer.

mediate halodioxolane, the amine, and a solvent in a pressure bottle on a steam bath. The periods of heating varied from 2 to 7 days. The products were isolated as hydrochlorides, oxalates, or methiodides.

In contrast to the above procedure for the preparation of 4-aminomethyl-1,3-dioxolanes, the preparation of the 2-aminomethyl analogs (Table V) required higher temperatures. For example, when a mixture of 2-bromomethyl-2-phenyl-1,3-dioxolane, morpholine, and benzene was heated in a pressure bottle on the steam bath for 7 days, the starting intermediates were recovered unchanged. The reaction was successfully conducted in the absence of solvent in sealed glass tubes heated at 140–150° for 24 hours. The products were also isolated as hydrochlorides, oxalates, or methiodides.

The condensation of Mannich bases with 1,2-glycols as a direct potential route to 2-aryl-2-(β-aminoethyl)-1,3-dioxolanes was studied. The condensation of β-morpholinopropiophenone hydrochloride with ethylene glycol was carried out with moderate success. Under similar reaction conditions, β-dimethylamino and α-methyl-β-dimethylaminopropiophenone hydrochlorides failed to yield the corresponding dioxolanes.

In preliminary experiments on isolated guinea pig ileum, compounds 1, 3, 5, 6, 8, 19, 22, 26, and 27 (Table IV), compounds 1, 6, and 10 (Table V), and 2-phenyl-2-(β-morpholinoethyl)-1,3-dioxolane hydrochloride exhibited low anticholinergic activity. Significant activity was observed with compound 9 (Table V).

In exploratory experiments on anesthetized dogs, compounds 3, 5, 13, 19, 27, 28, and 30 (Table IV), compounds 6 and 11 (Table V), and 2-phenyl-2-(β-morpholinoethyl)-1,3-dioxolane hydrochloride exhibited weak autonomic activity. Compound 9 (Table V) produced a fall in diastolic pressure and cardiac stimulation at an intravenous dose of 1 mg./Kg. A prolonged duration of action resulted on increasing the dosage to 5 mg./Kg.

EXPERIMENTAL[1]

The procedures used for the synthesis of the intermediate halodioxolanes and the final products are illustrated by the following examples.

2-Phenyl-2-(β-morpholinoethyl)-1,3-dioxolane Hydrochloride.—The compound was prepared according to the procedure of Salmi (10). A mixture of 38.5 Gm. (0.15 mole) of the required Mannich base salt (β-morpholinopropiophenone hydrochloride), 31.0 Gm. (0.5 mole) of ethylene glycol, 0.95 Gm. of *p*-toluenesulfonic acid monohydrate, and 200 ml. of toluene was refluxed for 3 hours in an assembly equipped with a Dean-Stark trap. The reaction mixture was rendered alkaline with 10% aqueous sodium hydroxide. The combined toluene layer and ether extracts of the aqueous phase were washed with water, dried, and concentrated to an oily residue *in vacuo*. Unreacted Mannich base was removed by vacuum distillation (b_3 77–83°). The distillation residue was converted to the hydrochloride with ethereal hydrogen chloride. The yield, after two recrystallizations from absolute ethanol, was 5.5 Gm. (12%), m.p. 220–221°.

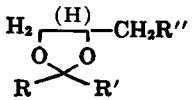
Anal.—Calcd. for $C_{15}H_{22}ClNO_3$: N, 4.67; Cl, 11.83. Found: N, 4.40; Cl, 11.66.

2-Bromomethyl-2-(*p*-phenylphenyl)-1,3-dioxolane.—(Table I, compound 11).—The compound was prepared according to the procedure of Salmi (10). A mixture of 27.5 Gm. (0.1 mole) of *p*-phenylphenacyl bromide, 62 Gm. (1 mole) of ethylene glycol, 0.95 Gm. of *p*-toluenesulfonic acid monohydrate, and 200 ml. of toluene was refluxed for 15 hours. The yield was 24 Gm. (76%), m.p. 79.5 to 80° after recrystallization from ethanol.

2-Benzyl-2-methyl-4-bromoethyl-1,3-dioxolane.—(Table II, compound 16.)—The compound was prepared according to the procedure of Bersin and Willfang (11, 12). A stirred solution of 20 Gm. (0.15 mole) of phenyl-2-propanone and 27.5 Gm. (0.2 mole) of epibromohydrin in 150 ml. of dry carbon tetrachloride was maintained at 0–5° for 2 hours during the dropwise addition of

[1] Melting points were determined on a Fisher-Johns or Nalge melting point apparatus and are uncorrected. Microanalyses are by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley.

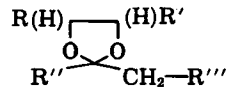
TABLE IV.—4-AMINOMETHYL-1,3-DIOXOLANE SALTS[i]



No.	R	R′	R″ (A,B,C)[a]	M.p., °C.	Yield, %	Formula	Analyses, % C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found	X[b] Calcd.	X[b] Found
1	*p*-BrC_6H_4	CH_3	NC_5H_{10}[c]·A	190–194	36	$C_{16}H_{23}BrClNO_2$	...	...	...	...	3.72	3.86	...	...
2	*p*-BrC_6H_4	CH_3	NC_4H_8O[d]·A	170–173	33	$C_{15}H_{21}BrClNO_3$	...	...	...	...	3.72	3.85	...	...
3	*p*-ClC_6H_4	CH_3	NC_5H_{10}·A	193–196	43	$C_{16}H_{23}Cl_2NO_2$	...	...	...	...	4.22	4.20	21.34	21.30
4	*p*-ClC_6H_4	CH_3	NC_4H_8O·B	204–206	41	$C_{17}H_{22}ClNO_7$	...	...	...	...	3.61	3.72	9.14	9.24
5	*p*-$CH_3OC_6H_4$	CH_3	NC_5H_{10}·C	181–187	56	$C_{18}H_{26}INO_3$	...	...	...	...	3.36	3.42	30.40	30.28
6	*p*-$CH_3C_6H_4$	CH_3	NC_5H_{10}·A	182–185	48	$C_{17}H_{26}ClNO_2$	...	...	...	...	4.49	4.61	11.37	11.59
7	*p*-$CH_3C_6H_4$	CH_3	NC_4H_8O·A	170–174	48	$C_{16}H_{24}ClNO_3$	...	...	...	...	4.46	4.63	11.30	11.41
8	C_6H_5	C_2H_5	NC_5H_{10}·A	197–202	69	$C_{17}H_{26}ClNO_2$	...	...	...	...	4.49	4.58	11.37	11.48
9	C_6H_5	C_2H_5	NC_4H_8O·A	186–189	46	$C_{16}H_{24}ClNO_3$	...	...	...	...	4.46	4.65	11.30	11.30
10	C_6H_5	*n*-C_3H_7	NC_5H_{10}·B	175–180	64	$C_{20}H_{29}NO_6$	63.31	63.21	7.70	7.77	3.69	3.74	...	...
11	C_6H_5	*n*-C_3H_7	NC_4H_8O·B	180–184	69	$C_{19}H_{27}NO_7$	59.83	60.01	7.14	7.07	3.67	3.50	...	...
12	C_6H_5	$CH(CH_3)_2$	NC_5H_{10}·A	220–222.5	61	$C_{18}H_{28}ClNO_2$	...	...	...	...	4.30	4.41	10.88	11.00
13	C_6H_5	$CH(CH_3)_2$	NC_4H_8O·A	204–207	52	$C_{17}H_{26}ClNO_3$	...	...	...	...	4.27	4.25	10.82	10.84
14	C_6H_5	$CH(CH_3)_2$	$N_2C_4H_9$[e]·2A	176–182	54	$C_{17}H_{28}Cl_2N_2O_2$	...	...	...	...	7.71	7.60	19.52	19.27
15	C_6H_5	$CH(C_2H_5)_2$	NC_5H_{10}·B	198–201	34	$C_{22}H_{33}NO_6$	64.84	64.82	8.16	7.94	3.44	3.23	...	...
16	C_6H_5	$CH(C_2H_5)_2$	NC_4H_8O·B	194–197	34	$C_{21}H_{31}NO_7$	61.60	61.71	7.63	7.51	3.42	3.27	...	...
17	C_6H_5	$CH(C_2H_5)C_4H_9$	NC_5H_{10}·B	140–144	45	$C_{24}H_{37}NO_6$	66.18	65.90	8.56	8.38	3.22	3.17	...	...
18	C_6H_5	$CH(C_2H_5)C_4H_9$	NC_4H_8O·B	145–147	46	$C_{23}H_{35}NO_7$	63.14	62.98	8.06	7.97	3.20	3.12	...	...
19	*n*-C_9H_{19}	CH_3	$N(C_2H_5)_2$·A	137–139	30	$C_{18}H_{36}ClNO_2$	...	...	...	...	4.17	4.45	10.55	10.29
20	$C_6H_5CH_2$	CH_3	NC_5H_{10}·B	128–131	59	$C_{19}H_{27}NO_6$	62.45	62.18	7.45	7.56	3.83	3.57	...	...
21	$C_6H_5CH_2$	CH_3	NC_4H_8O·B	137–140	48	$C_{18}H_{25}NO_7$	58.84	58.96	6.86	6.81	3.81	3.53	...	...
22	*p*-ClC_6H_4	C_6H_5	NC_5H_{10}·A	182–192	58	$C_{21}H_{25}Cl_2NO_2$	...	...	...	...	3.55	3.56	17.98	17.89
23	*p*-ClC_6H_4	C_6H_5	NC_4H_8O·A	181–185	52	$C_{20}H_{23}Cl_2NO_3$	...	...	...	...	3.53	3.70	17.89	18.05
24	*p*-$CH_3C_6H_4$	C_6H_5	NC_5H_{10}·A	190–195	11	$C_{22}H_{28}ClNO_2$	...	...	...	...	3.75	3.86	9.48	9.30
25	*p*-$CH_3C_6H_4$	C_6H_5	NC_4H_8O·B	182–184	33	$C_{23}H_{27}NO_7$	64.32	64.35	6.34	6.39	3.26	3.15	...	...
26	*p*-$CH_3C_6H_4$	*p*-$CH_3C_6H_4$	$N(C_3H_7)_2$·C	133–134	45	$C_{25}H_{36}INO_2$	...	...	...	...	2.75	2.85	24.91	24.66
27	*p*-$CH_3C_6H_4$	*p*-$CH_3C_6H_4$	NC_4H_8O·A	209–212	58	$C_{22}H_{29}ClNO_3$	...	...	...	...	3.59	3.66	9.09	9.03
28	2-C_4H_3S[f]	CH_3	$N(C_2H_5)_2$·B	140–143	25	$C_{15}H_{23}NSO_6$	...	...	...	...	4.06	3.78	9.28	9.52
29	2-C_4H_3S	CH_3	NC_4H_8O·B	129–131	62	$C_{15}H_{21}NSO_3$	...	...	...	...	3.90	4.13	8.92	8.60
30	2-C_4H_3S	C_6H_5	NC_4H_8O·C[g]	194–197	...	$C_{19}H_{24}INSO_3$[h]	...	...	...	...	2.96	2.91	26.81	26.60
31	2-C_4H_3S	C_6H_5	NC_5H_{10}·B	186–191	67	$C_{21}H_{25}NSO_6$	60.12	59.78	6.01	6.05	3.34	3.37	7.64	7.44

[a] A = hydrochloride; B = oxalate; C = methiodide. [b] Halogen or sulfur. [c] Piperidino. [d] Morpholino. [e] Piperazino. [f] 2-Thienyl. [g] The corresponding oxalate melted at 188–191°. [h] Calcd.: S, 6.77. Found: S, 6.60. [i] Reaction time: compounds 3, 19, 24, 27, 28—2 days; 26—2.5 days; 1, 12, 20—4 days; 22—4.5 days; 5—5 days; 10, 11, 13, 17, 18, 23, 25—7 days; all other compounds—6 days. Compounds 1–3, 8, 9, 19, 22–24, and 27 were recrystallized from isopropanol, the remainder from absolute ethanol.

TABLE V.—2-AMINOMETHYL-1,3-DIOXOLANE SALTS[j]



No.	R	R′	R″	R‴	M.p., °C.	Yield, %	Formula	Analyses, % N Calcd.	N Found	X,[a] Calcd.	X,[a] Found
1	H	H	C_6H_5	$N_2C_4H_9$[b]·2HCl	250[i]	23	$C_{14}H_{22}Cl_2N_2O_2$	8.72	8.68	22.07	21.89
2	H	H	*p*-BrC_6H_4	NC_5H_{10}[c]·HCl	224–227	48	$C_{15}H_{21}BrClNO_2$	3.86	3.98	...	...
3	H	CH_3	*p*-BrC_6H_4	$NC_5H_{10}·C_2H_2O_4$[d]	154–157	47	$C_{15}H_{24}BrNO_6$	3.26	3.30	18.57	18.82
4	H	H	*p*-ClC_6H_4	NC_5H_{10}·HCl	208–213	67	$C_{15}H_{21}Cl_2NO_2$	4.40	4.55	22.28	22.02
5	H	H	*p*-ClC_6H_4	NC_4H_8O·[e]HCl	200–205	38	$C_{14}H_{19}Cl_2NO_3$	4.37	4.49	22.15	22.08
6	H	CH_3	*p*-ClC_6H_4	NC_5H_{10}·HCl	189–191	80	$C_{16}H_{23}Cl_2NO_2$	4.20	4.18	21.34	21.24
7	CH_3	CH_3	*p*-ClC_6H_4	NC_5H_{10}·HCl	208–214	74	$C_{17}H_{25}Cl_2NO_2$	4.05	4.11	20.48	20.50
8	H	H	*p*-$C_6H_5C_6H_4$	$NC_5H_{10}·C_2H_2O_4$	179–180	30	$C_{23}H_{27}NO_6$[f]	3.39	3.50	...	...
9	H	H	*p*-$C_6H_5C_6H_4$	$NC_5H_{10}·CH_3I$	222–224	...	$C_{22}H_{28}INO_2$	...	...	27.27	27.60
10	H	H	*p*-$C_6H_5C_6H_4$	$NC_4H_8O·C_2H_2O_4$	195–198	42	$C_{22}H_{25}NO_7$[g]	3.37	3.08	...	...
11	H	H	C_8H_9[h]	$NC_5H_{10}·CH_3I$	219–223	31	$C_{18}H_{28}INO_2$	3.36	3.50	30.41	30.22
12	H	CH_3	C_8H_9	$NC_5H_{10}·CH_3I$	185–188	57	$C_{19}H_{30}INO_2$	3.25	3.35	29.42	29.38

[a] Halogen. [b] Piperazino. [c] Piperidino. [d] Oxalate. [e] Morpholino. [f] Calcd. C, 66.81; H, 6.58. Found: C, 66.98; H, 6.76. [g] Calcd.: C, 63.60; H, 6.07. Found: C, 63.30; H, 5.89. [h] 2,5-Dimethylphenyl. [i] With decomposition. [j] Compounds 2 and 5 were recrystallized from absolute ethanol-ether; 4, 6, and 7 from isopropanol; the remainder from absolute ethanol.

5.2 Gm. of stannic chloride in 50 ml. of carbon tetrachloride. The product was purified by distillation (b_3 110–111°).

2 - Methyl - 2 - (*p* - tolyl) - 4 - piperidinomethyl-1,3-dioxolane Hydrochloride.—(Table IV, compound 6.)—A solution of 11.5 Gm. (0.05 mole) of 2-methyl-2-(*p*-tolyl)-4-chloromethyl-1,3-dioxolane and 42.5 Gm. (0.5 mole) of piperidine in 50 ml. of benzene was heated in a pressure bottle on a steam bath for 6 days. The mixture was treated with 10% aqueous sodium hydroxide. The combined benzene layer and ether extract of the aqueous phase was concentrated *in vacuo*. The residue was converted to the hydrochloride and recrystallized from absolute ethanol. The yield was 7.5 Gm. (48%), m.p. 182–185°.

2 - (*p* - Phenylphenyl) - 2 - piperidinomethyl-1,3-dioxolane Methiodide.—(Table V, compound 9.)—A mixture of 11 Gm. (0.04 mole) of 2-bromomethyl-2-(*p*-phenylphenyl)-1,3-dioxolane and 8.5 Gm. (0.1 mole) of piperidine was heated at 150° in a sealed glass tube for 24 hours. The mixture was made alkaline with 10% aqueous sodium hydroxide and extracted with ether. The solvent and unreacted amine were removed *in vacuo*. Excess methyl iodide was added to an ether solution of the residue and allowed to stand at room temperature for 24 hours. The product was recrystallized from absolute ethanol, m.p. 222–224°.

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Wool Wax Emulsions

By H. I. SILVERMAN

Emulsions prepared with a wool wax isolate and its ethoxylated derivative have been formulated and studied. Rheological patterns were determined over a 12-month period and flow types characterized. Globular sizes were also determined and correlated with the observed stabilities (creaming rates) and emulgent concentrations. The inapplicability of Stokes' law in theoretical calculations of creaming rate has been shown. A stable emulsion may be produced by selecting as an emulsifying agent a material that will not form a true solution with either of the emulsion phases while exhibiting an affinity for or a greater solubility (colloidal sol) in the external phase.

WOOL WAX, its numerous fractions and synthetic derivatives, enjoy wide and varied applications in the cosmetic, pharmaceutical, chemical, and other fields. The chemical composition of this natural wax is varied and extremely complex. Briefly, it may be described as a mixture of a majority of straight chain and steroid waxy esters plus a minority of free fatty acids and alcohols. A comprehensive literature survey has been recently published (1, 2) which contains, in addition to references on chemistry, numerous sources of wool wax derivatives, both the purified fractions and chemically altered components of these fractions.

It is the purpose of this paper to report on the feasibility of using two of the aforementioned derivatives as emulgents. No single component of wool wax is seemingly responsible for its ability to act as an efficient w/o emulsifier. Rather, its value as an emulsifying agent is probably due to both the alcoholic and free fatty acid components (3,4).

Wool wax components may be chemically modified to increase their dipole moment by ethoxylation (5, 6). Polarity will increase in a linear proportion corresponding to the length of the coupled ethylene oxide polymer. This hydrophilic polymer, upon reaching a definite number of ethenoxy groups per molecule will allow the preparation of a water "soluble" colloidal sol from the originally water insoluble component. Due to the high molecular weight hydrocarbon components, true aqueous solutions are not possible although transparent dispersions can be produced. In addition, the increased hydrophilic property will tend to reverse the original emulsifying ability from w/o to o/w.

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MATERIALS

For this study an isolation product of wool wax and its ethoxylated derivative were selected as emulsifying agents. The isolation product was Lanfrax,[1] a water insoluble fraction, appearing physically as a stiff semisolid plastic material when at room temperature. This fraction is composed of a mixture of the higher melting range (49–51°) hard wax esters which have been removed from the lower melting components by a fractional solvent crystallization process (7).

Lanfrax WS55,[1] the ethoxylated derivative of the above-mentioned wax, is a nonionic surface-active and hydrophilic hard waxy material. Clear transparent colloidal sols may be prepared in water.

The balance of the emulsion systems was composed of distilled water, liquid petrolatum (viscosity 60 cps., sp. gr. 0.86), and a phenolic salt—sodium *o*-phenyl phenol[2] which functioned as a preservative.

EXPERIMENTAL

Formulations and Physical Data.—Lanfrax was used as the w/o emulsifying agent. At room temperature in mineral oil, clear colloidal sols may be produced using up to a 1% concentration by weight with uniform but cloudy dispersions possible at greater concentrations.

Lanfrax WS55 was used as the o/w emulsifying agent. Transparent colloidal sols in water may be produced at room temperature. The Tyndall cone phenomenon was observed with aqueous solutions of this ethoxylated wax derivative.

Experimental emulsions were similarly prepared by heating the external phase of each formulation to 65–70° and dissolving the emulsifying agent in it. The internal phases were likewise heated to the same temperature. The preservative was dissolved in each of the respective aqueous phases prior to emulsification. Emulsification was then carried

[1] Malmstrom Chemical Corp.
[2] Dow Chemical Co. (Dowicide A).

TABLE I.—FORMULATIONS

	Emulsion Number 02161A 100 Gm.	02161B 100 Gm.	02161C 100 Gm.
o/w Series			
1. Lanfrax WS55	3.0	5.0	8.0
2. Liq. petrolatum (visc. 60 cps.)	48.5	47.5	46.0
3. Distilled water	48.5	47.5	46.0
4. Preserve with sodium *o*-phenyl phenol[a] to give a final concn. in emulsion of 0.25% by weight.			
Relative consistency noted at time of manufacture.	"Thin Lotion"	"Medium Lotion"	"Heavy Lotion"
	Emulsion Number 02171A/1 100 Gm.	02171B/1 100 Gm.	02171C/1 100 Gm.
w/o Series			
1. Lanfrax	3.0	5.0	8.0
2. Liq. petrolatum (visc. 60 cps.)	48.5	47.5	46.0
3. Distilled water	48.5	47.5	46.0
4. Preserve with sodium *o*-phenyl phenol[a] to give a final concn. in emulsion of 0.25% by weight.			
Relative consistency noted at time of manufacture.	"Medium Lotion"	"Heavy Lotion"	"Very Heavy Lotion"

[a] Dowicide A.

out by placing the external phase, containing the emulsifying agent, in a Waring Blendor connected to a powerstat (set to a dial reading of 75) and slowly adding the internal phase. After mixing 2–3 minutes, the emulsion, (which formed immediately in each case) was removed from the blender, allowed to cool to approximately 60°, and then passed twice through a piston homogenizer. Several batches of the respective emulsions (500 Gm. each) were processed by this method and were used in studying both stability and rheological patterns.

Formulations are set forth in Table I, and as can be noted each of the emulsion types was prepared at emulgent concentration of 3, 5, and 8%, respectively, by weight.

Stability Studies.—A 100-ml. quantity of each formulation was poured into separate matched glass cylinders of 2.5 cm. i.d. Emulsion height in these tubes was 20.5 cm. Apparent viscosities of the external emulsion phases, each of which contained the listed content of emulsifying agent, was determined at minimum shear by a Brookfield Viscometer, model LVT, and recorded in Table II. Specific gravities of the above-described phases were determined by the pycnometer method at 25° and are likewise recorded in Table II. At predetermined intervals emulsions were microscopically examined in order to record the globular size of the dispersed

TABLE II.—VISCOSITY AND SPECIFIC GRAVITY DATA DETERMINED AT 25°C.

External[a] Phase	Viscosity[b] at Minimum Shear, cps.	Specific Gravity
o/w Series		
02161A	10	1.01
02161B	20	1.01
02161C	300	1.02
w/o Series		
02171A/1	100	0.863
02171B/1	800	0.864
02171C/1	5,000	0.866

[a] External phase containing Lanfrax WS55 in o/w series and Lanfrax in w/o series. [b] Brookfield Viscometer, model LVT.

TABLE III.—AVERAGE GLOBULAR DIAMETER AND RELATIVE DISTRIBUTION

	Days After Manufacture 5	30	60
o/w Series			
02161A	2–14 μ, wide variation in globular size 6 μ av.	3–15 μ, wide variation in globular size 6 μ av.	6 μ av., moderate packing of globules
02161B	2–3 μ, globules are fairly uniform	1.5 to 3 μ, globules fairly uniform and tightly packed	2 μ av., globules are uniform and tightly packed
02161C	0.4–0.5 μ, globules are uniform	0.5 μ, globules both uniform and tightly packed	0.3–0.5 μ (av. 0.5 μ), globules are uniform and tightly packed
w/o Series			
02171A/1	2–4 μ, with variation in globular size	1.5–6 μ, with variation in globular size	1.5–7 μ (av. 3 μ), globules not uniform and loosely packed
02171B/1	1–6 μ, with variation in globular size	1–6 μ, with variation in globular size	1–6 μ (av. 2 μ), globules not uniform and loosely packed
02171C/1	1.5–3 μ, globules are uniform	1–3 μ, globules are uniform and tightly packed	1–3 μ (av. 1.5 μ), globules are uniform and tightly packed

TABLE IV.—CREAMING[a] AND PHASE[b] SEPARATION, OBSERVED[c] AND THEORETICAL

	Time, Days								
	5		10		25		60		120
Formula	Actual	Calcd.-C	Actual	Calcd.-C	Actual	Calcd.-C	Actual	Calcd.-C	Actual
02161A	1.5-LC	12.7	2.8-LC	25.4	4.4-LC	63.5	4.8-LC	152.4	4.8-LC
02161B	0.1-LC	0.709	0.6-LC	1.42	1.0-LC	3.54	1.2-LC	8.50	1.8-LC
02161C	0	0.003	0	0.006	0	0.016	0	0.038	0
02171A/1	0.05-UP	0.030	0.05-UP	0.059	0.2-UP	0.148	1.1-UP	0.356	2.8-UP
02171B/1	0	0.016	0	0.033	0	0.082	0.05-UP	0.198	0.90-UP
02171C/1	0	0.001	0	0.003	0	0.007	0	0.016	0

[a] In cm. of zone height of separated emulsion containing a *lesser* concentration of dispersed phase; column part-L is bottom, U is top; C is creaming. [b] In cm. of zone height; column part-L is bottom, U is top; P is phase. [c] 100 ml. of each emulsion placed into glass tubes of 2.5 cm. i.d., height of emulsion in tubes was 20.5 cm.

phase. Droplet size may be used as an aid both in stability prediction (8) and creaming rate calculations by the use of Stokes' law.

With the use of outlined techniques as a guide (9, 10), diameters of the dispersed particles were determined and then recorded in Table III. A Levy Hemacytometer with Fuchs-Rosenthal ruling and a calibrated ocular micrometer disk were employed for the counting procedure. Approximately 1000 globules in each emulsion were microscopically measured. Brownian movement was noted in each of the experimental emulsions and was especially significant in the w/o series.

Creaming and phase separation were noted by measuring and recording their respective zone heights at definite intervals. These measurements coincided with times of globular examination.

Observations on the former two phenomena were recorded over a period of 4 months (Table IV). Separation zone placement in the stability columns is also recorded.

Creaming Rate Calculation.—Certain of the aforementioned data (diameter of dispersed globule, viscosity, and specific gravity) may be used in theoretical calculations of the creaming rate by applying Stokes' law. The actual zone distance through which the dispersed globules moved during the stability observation has been compared to the theoretical distance of movement calculated by Stokes' law (Table IV). As an example of a theoretical calculation the rate of globule movement (creaming) is herewith calculated for emulsion 02161A for a 5-day period of standing.

1. Viscosity at minimum shear of external phase containing emulsifying agent..... 0.1 poise
2. Specific gravity of external phase containing emulsifying agent..... 1.01
3. Specific gravity of the dispersed phase (liquid petrolatum)..... 0.86

$$\text{Stokes' eq.: } V = \frac{d^2(P_1 - P_2)g}{18\,n}$$

where V = velocity of sedimentation; d = diameter of globules of the dispersed phase; P_1 = specific gravity of the dispersed phase; P_2 = specific gravity of the dispersion medium; g = gravitational constant; n = viscosity of the dispersion medium.

Solving for V

$$V = \frac{(6 \times 10^{-4})^2\,(0.86 \times 1.01)\,980}{18 \times 0.1}$$

$$= 2.94 \times 10^{-5} \text{ cm./sec.}$$

To determine the rate of globular rise in a 5-day period, then multiply by a factor of 4.32×10^5. Since there are 60 seconds in a minute $\times$ 60 minutes in an hour = 3600 seconds. In 24 hours there are 86,000 seconds and in 5 days there would be 432,000 seconds: $(2.94 \times 10^{-5}) \times (4.32 \times 10^5) = 12.70$ cm./5-day rate of globule rise since the density of the dispersed phase is less than that of the continuous phase.

With the exception of theoretical calculations at the 120-day period, both actual and calculated zones have been recorded (Table IV).

TABLE V.—APPARENT VISCOSITIES O/W SERIES[a]

		Viscosity, cps.			
Emulsion age		5 Days	30 Days	60 Days	12 Mo.
Formula			02161A		
Speed, r.p.m. (Spindle no. 2)	60	75	73	70	58
	30	95	90	85	74
	12	148	138	125	113
	6	210	200	175	180
	3	330	300	280	270
	1.5	500	400	360	414
Formula			02161B		
Speed, r.p.m. (Spindle no. 2)	60	320	308	287	251
	30	460	440	410	372
	12	790	775	725	664
	6	1265	1220	1170	1094
	3	2060	2050	1950	1823
	1.5	3480	3480	3240	3106
Formula			02161C		
Speed, r.p.m. (Spindle no. 3)	60	1930	1750	1600	1530
	30	2948	2700	2500	2400
	12	5500	5000	4650	4500
	6	8900	8200	7800	7500
	3	14,920	14,000	13,200	12,800
	1.5	25,280	24,400	23,200	22,000

[a] Brookfield Viscometer, model LVT, room temperature 23–25°C.

TABLE VI.—APPARENT VISCOSITIES W/O SERIES[a]

		Viscosity, cps.			
Emulsion age		5 Days	30 Days	60 Days	12 Mo.
Formula			02171A/1		
Speed, r.p.m. (Spindle no. 3)	60	830	780	570	750
	30	1056	1000	700	984
	12	1550	1450	900	1570
	6	2300	2100	1300	2400
	3	3600	3200	1800	3880
	1.5	5600	5600	3040	6560
Formula			02171B/1		
Speed, r.p.m. (Spindle no. 4)	60	5300	3700	2350	2100
	30	7600	5600	3260	3000
	12	13,000	9500	5400	5000
	6	22,000	15,000	8500	8000
	3	33,400	26,000	14,400	14,000
	1.5	58,000	46,000	25,200	22,400
Formula			02171C/1		
Speed, r.p.m. (Spindle no. 4)	60	...	8950	4700	3500
	30	15,400	14,200	6800	5000
	12	27,500	26,000	11,500	9500
	6	47,000	45,000	19,500	16,000
	3	84,000	80,000	35,000	28,000
	1.5	140,000	144,000	64,000	52,000

[a] Brookfield Viscometer, model LVT, room temperature 23–25°C.

Apparent Emulsion Viscosities.—Emulsions were stored in 500-ml. quantities in suitable containers which were inverted three times prior to instrumental analysis of viscosities. In addition, at 3-day intervals for a 2-month period only, containers were opened, closed, and shaken vigorously. As was expected, all of the emulsions behaved similarly when their flow properties were studied. All were non-Newtonian and exhibited plastic flow. Apparent viscosities at time intervals have been recorded in Tables V and VI. Corresponding to these tables and using a semilog scale, Fig. 1 plots viscosity characteristics of the emulsions when they are subjected to increasing shearing rates. Finally, Figs. 2 and 3 illustrate a pseudoplastic type of non-Newtonian flow for all of the emulsions as well as an apparent yield value. The latter may be easily extrapolated from the base of each of the flow curves. Shearing rate was recorded as r.p.m. and shearing stress (torque) as scale readings of the Brookfield Viscometer, model LVT. It is most important when studying rheological characteristics of a non-Newtonian system to record all variables. In this experiment, temperature, emulsion age, Brookfield spindle size, rate and shear, and shearing stress or torque (Brookfield scale readings) have been recorded in order to classify properly rheological behavior.

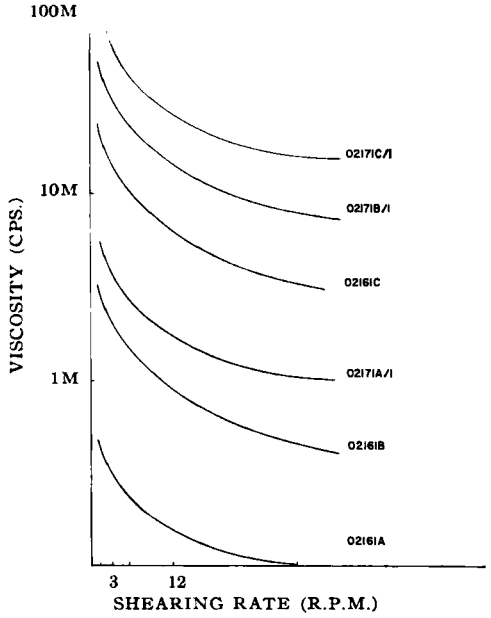

Fig. 1.—Rheogram characterizing type of flow. Sample age 5 days.

DISCUSSION AND CONCLUSIONS

In general, over the time period during which data were collected and recorded in this study (12 months), all emulsions of both types were stable in

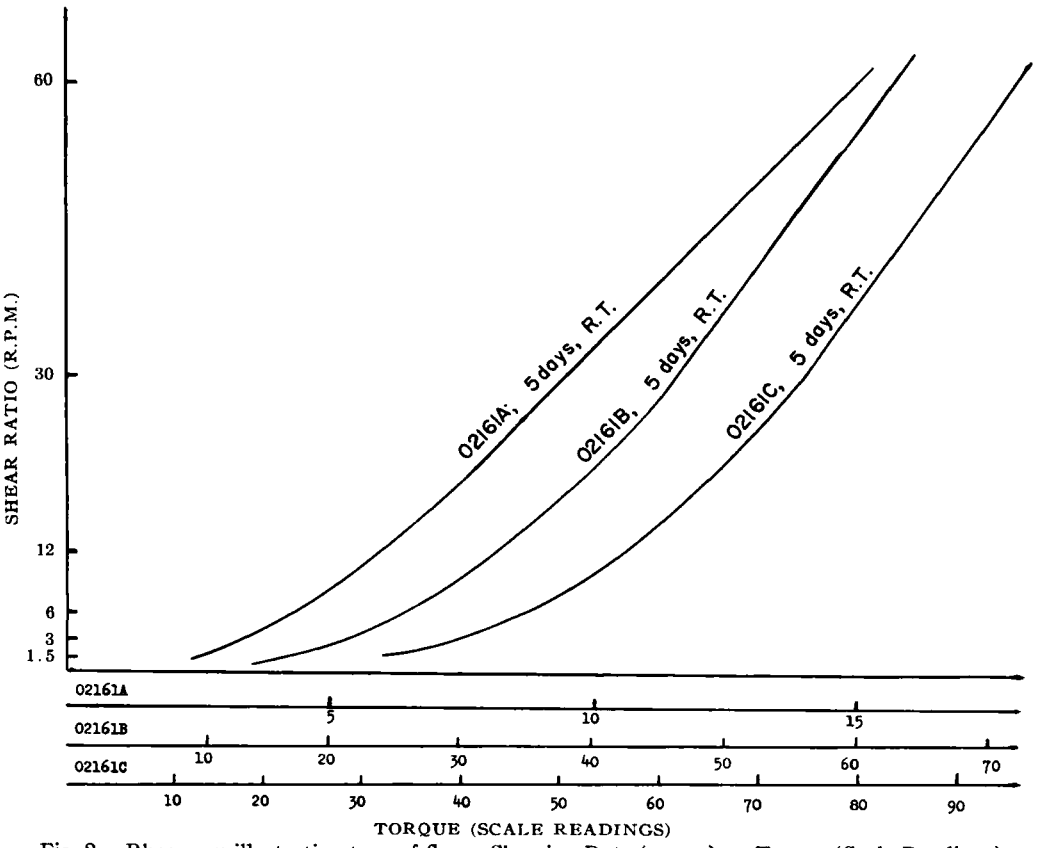

Fig. 2.—Rheogram illustrating type of flow. Shearing Rate (r.p.m.) *vs.* Torque (Scale Readings).

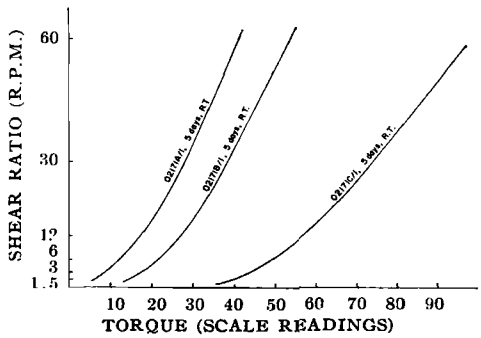

Fig. 3.—Rheogram illustrating type of flow. Shear Rate (r.p.m.) *vs.* Torque (Scale Readings).

regard to cracking. In the o/w series some creaming occurred when the concentration of emulsifying agent (Lanfrax WS55) was held at 3 and 5%, respectively.

As would be expected with the moderate creaming exhibited, simple container inversion was sufficient to reblend these formulas. At an 8% concentration a uniformly stable product resulted with good stability (both observed and when theoretically calculated by a commonly accepted procedure). The latter emulsions' optimum stability is interesting in light of common practice to use thickening agents and emulsifier blends to stabilize emulsion systems. Good stability was observed in all of the w/o emulsions, with again optimum stability at the higher emulgent concentrations. The greater viscosity of the external phase in this series assisted in stabilization. It is interesting to note that Lanfrax as a w/o emulsifying agent exhibits dual functions of thickener as well as emulsifier.

All of the rheograms show expected patterns. Flow may be characterized as pseudoplastic with yield values.

It is interesting to note that the o/w series exhibited a less changeable viscosity pattern than the w/o group. Viscosity drop was generally progressive for the first 2 months, and leveling off afterward. The repeated shaking during the initial 60 days may have contributed further to a weakening in gel pattern. Finally, the omission of an antioxidant from the w/o series and a possible interaction between the phenolic preservative and wax esters could also have played some part in the change of rheological characteristics.

Globular size was generally unchanged over the 2-month observation period. As the degree of creaming (when it occurred) was only moderate, this is not unexpected.

Contrary to Becher (8), Brownian movement was readily noted in all emulsions and this phenomenon was most apparent in the w/o series. That the w/o emulsions were, in general, somewhat less uniform in globular size may be a contributing factor. At the same time, however, the varied droplet size should also tend to increase stability by improved packing. Evidence of this assumption may be noted by examining data in Tables II and III. Observable instability proceeded at a lower rate in the w/o series than in the o/w emulsions.

While a higher viscosity of an emulsion external phase will improve shelf life through retardation of the creaming rate, such improvement in stability is necessarily coupled with globular packing.

The occasional application of Stokes' law to emulsion systems would appear to have several faults. To apply this law (which is suitable for highly dilute (under 0.5%) suspensions) to emulsion systems is seemingly in error. Few of the calculated rates of globular rise correspond to the observed rate. In addition, the following important considerations are neglected: globular size variation; dispersed phase charge (zeta potential); tendency of globules to coalesce; free globular movement hindered due to close packing; Brownian movement affecting globules of 1 μ and less diameter; temperature; concentration of emulsifying agent; and viscosity of dispersed phase may be non-Newtonian.

Stokes' law, in respect to emulsions, should be reserved simply to aid in determining the direction of creaming. It is obvious that a perfectly stable emulsion is one which does not exhibit creaming and/or phase separation. Of course the final stage of instability is where the system eventually reverts to completely separate phases (thermodynamically stable state). Since an emulsion is a potentially changeable system, its natural tendency is toward reversion. Reversion may be delayed through:

A Use of suitable equipment to produce dispersed globules with diameters of 1 $\pm$ 0.5 μ;

B Maintaining the specific gravity of the emulsified phases as close together as possible; and

C Use of an emulsifier to form a suitable interfacial film (which functions to prevent globular coalescence) as well as to cause an increase in the viscosity of the external phase.

This film may be produced by an agent having a greater solubility (colloidal sol) in one phase than another but yet will not form a true solution in either one.

Other factors such as temperature, viscosity, and phase volume ratio, play a part; however, the aforementioned points are those of greatest significance in emulsion stabilization.

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Tumor Inhibitors II

Alkaloids of *Ervatamia dichotoma*. Isolation, Crystallization, and Pharmacological Properties of Coronaridine

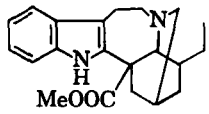
By S. MORRIS KUPCHAN, ALAN BRIGHT, and EDWARD MACKO†

Extraction of the fruit, root bark, and stem bark of *Ervatamia dichotoma* (Roxb.) Blatter from Mangalore, Mysore State, afforded substantial yields of alkaloids. Column chromatography of the petroleum ether-extractable alkaloids yielded coronaridine, which was obtained in crystalline form for the first time. Preliminary pharmacological testing in a variety of pharmacologic procedures revealed that the alkaloid mixtures and coronaridine possess interesting biologic activities.

ERVATAMIA DICHOTOMA (Roxb.) Blatter (syn. *Tabernaemontana dichotoma* Roxb.) is a small apocynaceous tree common in India and Ceylon. The seeds have been known to be poisonous and to possess narcotic and purgative properties; the leaves, bark, and milky sap have also been shown to be purgatives (1). The bark of *Ervatamia dichotoma* was studied by Subbaratnam, who reported the presence of alkaloids (0.01%) and sterols (0.2%) (2).

The work reported herein concerns the alkaloids of *Ervatamia dichotoma* from Mangalore, Mysore State. Initial petroleum ether extraction of the fruit yielded 0.7% of crude alkaloid; the root bark, 0.18%; and the stem bark, 0.09%. Subsequent methanol extraction of the fruit yielded 0.39% of crude alkaloids; the root bark, 0.49%; and the stem bark, 0.32%. The petroleum ether-extractable alkaloids of the fruit showed CNS depressant and hypotensive activities; the methanol-extractable fraction showed reproducible antitumor activity against 9KB cell culture.[1] Fractionation studies of the methanol-extractable alkaloids are in progress and will be described in a later communication.

Column chromatography on neutral alumina of the petroleum ether-extractable alkaloids of the fruits gave a crystalline material, m.p. 92–93°, $[\alpha]_D^{25}$ −34°, identified as coronaridine (I) (3).



I

Coronaridine does not appear to have been obtained in a crystalline form previously. The hydrochloride of the alkaloid showed m.p. 210–211° (decompn.) $[\alpha]_D^{25}$ −8°. The hydrochloride was found to be identical with coronaridine hydrochloride (3) by comparisons of infrared and ultraviolet spectra (4) and of X-ray diffraction patterns.

EXPERIMENTAL

Melting points, determined on a Hershberg apparatus, were corrected for stem exposure. Infrared spectra were determined on a Baird model B double beam infrared recording spectrophotometer. Ultraviolet absorption spectra were determined on a model 11 MS Cary recording spectrophotometer. All solutions were reduced in volume by heating at a temperature not exceeding 50–55° under reduced pressure. Chromatographic fractions were combined where indicated by paper chromatography and infrared spectroscopy. Petroleum ether of b.p. 40–60° was used throughout unless otherwise specified.

Extraction Procedure.—Dried powdered fruit of *Ervatamia dichotoma* (gathered in Mangalore, Mysore State, in April 1958)[2] (460 Gm.) was extracted with petroleum ether (2.5 L.) in a Soxhlet apparatus for 14 hours. The alkaloids were extracted with 4% hydrochloric acid and the acid solution was neutralized with ammonium carbonate and extracted with ether. Evaporation left an amorphous powder (3.0 Gm., fraction A). The dried marc remaining from the petroleum ether extraction was next extracted continuously with hot methanol (3 L.) with a fresh charge of solvent at the end of 2 days. The methanolic extract was evaporated to dryness under reduced pressure and the residue was triturated with 4% hydrochloric acid solution for 3 days. The process was repeated twice more with fresh charges of 4% hydrochloric acid. The suspension was filtered, and the acid solution was neutralized with ammonium carbonate and extracted with ether to yield a crude alkaloidal residue (1.78 Gm., fraction B).

Dried, powdered root bark (2.7 Kg.) was extracted as described above for the fruit. The petroleum

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Part I in the series: S. M. Kupchan and R. W. Doskotch, *J. Med. Pharm. Chem.*, **5**, 657(1962).

[1] The evaluation of the 9KB assay results by the Cancer Chemotherapy National Service Center in sequential testing is such that a material is considered active if the ED50 ≦ 20 mcg./ml. Assays were performed by CCNSC by the procedures described in Cancer Chemotherapy Reports, **25**, 1 (1962).

[2] We thank Dr. C. B. Sulochana, University Botany Laboratory, Madras, India, for confirming the identity of the plant, and Rajaranga and Co., Madras, India, for gathering and forwarding the dried plant material to us.

ether extract yielded 5.0 Gm. of crude alkaloidal residue (fraction C); the methanol extract yielded 13.30 Gm. (fraction D).

Dried, powdered stem bark (2.08 Kg.) yielded 1.9 Gm. of petroleum ether-extractable alkaloids (fraction E) and 6.67 Gm. of methanol-extractable alkaloids (fraction F).

Isolation of Coronaridine: Fraction A.—The crude mixture (3.0 Gm.) was dissolved in benzene and chromatographed on "neutral" alumina (Woelm. grade 1, 90 Gm.). The fractions eluted with benzene alone were combined. The chromatography on alumina was repeated as above, and the fractions eluted with benzene yielded crystalline residues upon evaporation. Repeated recrystallization from petroleum ether (b.p. 60–68°) gave colorless needles, 560 mg., m.p. 92–93°, $[\alpha]_D^{25} -34°$ (c 1.00, $CHCl_3$); $\lambda_{max.}^{alc.}$ 226 mμ (34,800), 286 mμ (8300), and 294 mμ (7800), $\lambda_{max.}^{CS_2}$ 2.82, 2.86, 3.33, 3.45, 5.82, 6.17, 7.04, 8.00, 8.51, 8.77, 9.26, and 9.80 μ.

Treatment of the crystalline alkaloid with a saturated ethereal solution of hydrogen chloride afforded an amorphous hydrochloride salt. Recrystallization from acetone yielded a microcrystalline solid, m.p. 210–211° (decompn.), $[\alpha]_D^{25} -8°$ (c 1.00, MeOH). The ultraviolet and infrared spectra were identical to those recorded for coronaridine hydrochloride (4). The X-ray diffraction pattern was found to be identical with an authentic sample of the hydrochloride of m.p. 235° (decompn.).[3]

PHARMACOLOGICAL RESULTS

Coronaridine was tested in a variety of pharmacologic procedures and found to possess biological activity. Thus, ptosis was produced in the mouse after doses as low as 5 mg./Kg. intraperitoneally. Higher doses, up to 100 mg./Kg., caused hypotonia, intermittent periods of increased and decreased activity, lacrimation, salivation, bradypnea, and finally tremors at the high dose level. Orally, coronaridine produced slight depression of spontaneous motor activity, lacrimation, bradypnea, and slight ptosis after doses as low as 20 mg./Kg. Lethal effects were apparent at 500 mg./Kg.

In the larger unanesthetized animal, coronaridine exhibited a variety of actions which were not consistent with different species. For instance, doses of 20 mg./Kg. intraperitoneally in the cat caused emesis, vocalization, ataxia, and death. A dose of 10 mg./Kg. intravenously in the dog produced excitation, ataxia, fear, ptosis, and restlessness, and yet in the monkey no overt effects were observed after doses of 10 mg./Kg. intravenously or 100 mg./Kg. orally. This phenomenon of species sensitivity to drugs is not uncommon. Many potentially interesting drugs have been very active in the lower animal but less active or inactive in the higher animal.

Coronaridine was found to possess analgetic activity in the rat using both the D'Amour Smith and Randall-Selitto test procedures over a range of doses from 15 to 100 mg./Kg. orally. In addition, this compound demonstrated weak but significant activity against foot shock induced rage in mice. This test can be used to uncover compounds which may possess mid-range tranquilizing activity.

Other tests were carried out in an effort to determine the possible central nervous system action of the compound. It was found that coronaridine did not potentiate hexobarbital anesthesia, that it failed to antagonize the lethal effect of amphetamine in aggregate mice, and did not produce an anticonvulsant action against maximal electroshock in mice.

The principal action of coronaridine on the blood pressure was depressor. In the anesthetized cat, this compound produced falls in pressure and a concomitant depression of respiration after intravenous doses as low as 0.5 mg./Kg. Lethality occurred at a dose of 10 mg./Kg.

In summary, coronaridine produced signs of autonomic as well as central nervous system activity when tested for biological action in animals. It produced analgesia and was effective in suppressing foot shock induced rage in mice. Toxicity appeared to be associated with respiratory depression in the anesthetized cat. Coronaridine was inactive against 9KB cell culture.

[3] We thank Dr. Norbert Neuss of the Lilly Research Laboratories, Indianapolis, Ind., for the X-ray pattern comparison of our material with authentic coronaridine hydrochloride.

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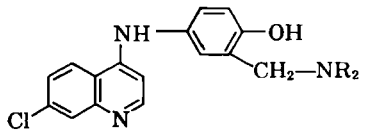
Antimalarial Agents VIII. Synthesis of Amopyroquine

By W. LEWIS NOBLES, RAYMOND F. TIETZ, YUN SHIK KOH, and J. H. BURCKHALTER†

Synthesis of the new parenteral antimalarial amopyroquine and analogs is described.

A BRIEF chemical study was designed to provide analogs of the antimalarial agent amodiaquine (I) hydrochloride U.S.P. (1) in which the diethylamino group was replaced by pyrrolidino, hexamethylenimino, and 4-methylpiperazino (structures II, IV, and V, respectively). The piperidino



		—NR_2
I	(amodiaquine)	$-N(CH_2CH_3)_2$
II	(amopyroquine)	$-N$
III		$-N$
IV		$-N$
V		$-N\langle\rangle NCH_3$

analog III has been previously described (1). The ethyl groups of I are free to move, while the movements of the corresponding groups of II, III, IV, and V are restricted owing to the cyclic structures. Models readily show the three-dimensional nature of the cyclic amines piperidine, hexamethylenimino, and N-methylpiperazine, while all the carbon atoms of pyrrolidine occupy the same plane. Also, V contains an additional ionic center as seen in the second basic nitrogen. In view of these considerations, the compounds were desired for a study of relative antimalarial activity and usefulness.

In order to obtain amopyroquinone (II),[1] *p*-acetamidophenol, pyrrolidine, and aqueous formaldehyde were subjected to Mannich conditions (1). When the intermediate *p*-acetamido-α-pyrrolidino-*o*-cresol was not obtained in crystalline form, the oily substance was hydrolyzed by acid and the unisolated diamine condensed with 4,7-dichloroquinoline to give 4-(7-chloro-4-quinolylamino)-α-pyrrolidino-*o*-cresol (amopyroquine II) in 78% yield. Compounds IV and V were similarly prepared by substitution of hexamethylenimine and N-methylpiperazine for pyrrolidine. In the case of IV, intermediate *p*-acetamido-α-hexamethylenimino-*o*-cresol could be isolated as the crystalline monohydrochloride in 51% yield.

An acridine analog of amopyroquine was obtained by substitution of 6,9-dichloro-2-methoxyacridine for 4,7-dichloroquinoline in the preparative procedure of II.

Pharmacological Results.[2]—Compound II (amopyroquine) is 37 times as active as quinine (*Plasmodium lophurae* in chicks) or equiactive with amodiaquine (2). Toxicity studies reveal that amopyroquine is considerably less toxic than amodiaquine, suggesting the former as a potential antimalarial for parenteral use (2). It has been found to be effective in human malaria (3) and is especially recommended as an agent of choice for intramuscular administration (4).

Compound III is 10 times as active as quinine (*P. lophurae* in ducks) (6), while IV is 3.5 times as active as quinine, V is 1.5 and the 6-chloro-2-methoxy-9-acridyl analog of amopyroquine is 5 (*P. lophurae* in chicks).

Conclusions drawn from the limited biological data in animals are that a total of four carbon atoms in the NR_2 grouping of structures I to V affords optimum antimalarial effectiveness; and if the carbons are rigidly held in the same plane as in amopyroquine, systemic toxicity is low.

EXPERIMENTAL

4-(7-Chloro-4-quinolylamino)-α-pyrrolidino-*o*-cresol (II).—A mixture of 45.3 Gm. (0.3 mole) of *p*-acetamidophenol, 25 ml. (0.3 mole) of 37% formaldehyde solution, 21.3 Gm. (0.3 mole) of pyrrolidine, and 75 ml. of 95% ethyl alcohol was refluxed for 2½ hours. When a solid product was not isolated, the solvent was removed *in vacuo* to leave a thick, sirupy residue. One-third of the residue was refluxed for 1 hour with 50 ml. of 20% hydrochloric acid. The solution was cooled and treated with 6*N* sodium hydroxide until just acidic to Congo red. After the addition of 19.8 Gm. (0.1 mole) of 4,7-dichloroquinoline, the mixture was refluxed for 2 hours. The solution was cooled and made basic with 28% ammonium hydroxide to give 27.5 Gm. (78% yield) of light yellow product, m.p. 186–191° dec. A sample was prepared for analysis by repeated recrystallization from isopropyl alcohol-water, m.p. 196–198° dec.

Anal.—Calcd. for $C_{20}H_{20}ClN_3O$: C, 67.88; H, 5.69. Found: C, 67.60; H, 5.76.

Dihydrochloride of II.[3]—Crude II free base was dissolved in warm isopropyl alcohol, treated with charcoal and then with an excess of dry hydrogen chloride to precipitate a yellow crystalline dihydrochloride, m.p. 288–290° dec. Two recrystallizations from isopropyl alcohol elevated the melting point to 299–300° dec.

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Paper VII: Burckhalter, J. H., Edgerton, W. H., and Durden, J. A., *J. Am. Chem. Soc.*, **76**, 6089(1954).

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[1] Marketed as Propoquin by Parke, Davis and Co., Detroit, Mich.

[2] Dr. P. E. Thompson, of Parke, Davis and Co., Research Department, Ann Arbor, Mich., has kindly furnished data on antimalarial studies in chicks.

[3] Data kindly furnished by Dr. Robert F. Meyer, Research Department, Parke, Davis and Co., Ann Arbor, Mich.

Anal.—Calcd. for $C_{20}H_{20}ClN_3O \cdot 2HCl$: C, 56.28; H, 5.20; Cl (ionic), 16.62. Found: C, 55.95; H, 5.36; Cl, 16.26.

4-(6-Chloro-2-methoxy-9-acridylamino)-α-pyrrolidino-o-cresol Dihydrochloride Sesquihydrate.—One-third of the residue from the preparation of II was treated as in that experiment except that 27.8 Gm. (0.1 mole) of 6,9-dichloro-2-methoxyacridine replaced 4,7-dichloroquinoline. The hot reaction mixture was filtered in order to remove insoluble 6-chloro-9-(4-hydroxyanilino)-2-methoxyacridine. Cooling the filtrate gave 30 Gm. (56% yield) of dihydrochloride, m.p. 245–249° dec. It was recrystallized from alcohol-acetone, m.p. 252–253° dec.

Anal.—Calcd. for $C_{25}H_{24}ClN_3O_2 \cdot 2HCl \cdot 1^1/_2H_2O$: C, 56.25; H, 5.47. Found: C, 56.54; H, 5.37.

p-Acetamido-α-hexamethylenimino-o-cresol Monohydrochloride.—A mixture of 15.1 Gm. (0.1 mole) of *p*-acetamidophenol, 9.9 Gm. (0.1 mole) of hexamethylenimine (5), 3 Gm. (0.1 mole) of paraformaldehyde, and 25 ml. of alcohol was heated to boiling for 4 hours. When cooling gave no precipitate, solvent was removed by distillation under reduced pressure. The residual dark oil was dissolved in ether, and excess alcoholic hydrogen chloride added to precipitate a yellow viscous oil. After decantation of the ether, solution in hot isopropyl alcohol and cooling gave 15.3 Gm. (51% yield) of product, m.p. 192–194°. Recrystallization from the same solvent elevated the melting point to 194.5 to 195°.

Anal.—Calcd. for $C_{15}H_{22}N_2O_2 \cdot HCl$: C, 60.29; H, 7.76. Found: C, 60.46; H, 7.96.

4-(7-Chloro-4-quinolylamino)-α-hexamethylenimino-o-cresol (IV).—A mixture of 14.3 Gm. (0.0478 mole) of *p*-acetamido-α-hexamethyleniminо-*o*-cresol monohydrochloride and 20 ml. of 20% hydrochloric acid was heated at reflux temperature for 1 hour. Sodium hydroxide solution was added to the cooled solution until it was barely acidic to Congo red. After the addition of 9.5 Gm. (0.048 mole) of 4,7-dichloroquinoline, the mixture was heated on a steam bath for 2 hours. Then, with cooling, it was made basic to litmus with 10% sodium hydroxide. The precipitated yellow solid was recrystallized twice from benzene to give 3.8 Gm. (21% yield) of IV, m.p. 212–214° dec.

Anal.—Calcd. for $C_{22}H_{24}ClN_3O$: C, 69.19; H, 6.33. Found: C, 69.31; H, 6.35.

It was found that following the procedure for II, which does not entail isolation of a purified acetamido Mannich phenolic base, actually led to a better yield of IV (45%).

4-(7-Chloro-4-quinolylamino)-α-(4-methyl-1-piperazinyl)-o-cresol (V) Trihydrochloride Dihydrate.—Procedure for II was followed; however, the free base was extracted with chloroform instead of being isolated. The extract was washed with water, dried over potassium carbonate, and treated with excess alcoholic hydrogen chloride. The addition of acetone completed precipitation of product. Recrystallization from alcohol gave 28 Gm. (53% yield) of bright yellow crystalline powder, m.p. 240–300° dec. It is very soluble in water.

Anal.—Calcd. for $C_{21}H_{23}ClN_4O \cdot 3HCl \cdot 2H_2O$: C, 47.73; H, 5.72. Found: C, 47.67; H, 5.93.

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Antifungal Properties of Perfume Oils

By JASPER C. MARUZZELLA

The antifungal properties of 30 perfume oils were tested by allowing the organisms to grow in varying concentrations of the oils *in vitro*. Fifteen of the perfume oils inhibited the growth of all test organisms at concentrations ranging from 1:500 to 1:13,000. The remaining 15 oils inhibited some of the test organisms at concentrations up to 1:11,000. Oil of rose no. 81412 otto type, crab apple blossom, and rose briar were found to possess marked antifungal properties. The dermatophytes were extremely susceptible to many of the perfume oils at minute concentrations.

PERFUME OILS were demonstrated to possess remarkable fungicidal properties when studied by the filter paper disk method (1). However, the oils were tested in the undiluted form at which strength they are rarely used in cosmetics and medicaments. Small amounts of perfumery materials are added to toilet articles and dermatological products in an attempt to render the item more fragrant and to mask unpleasant odors. Whether such minute concentrations possess germicidal properties has been suggested (2) but not experimentally established. This investigation was undertaken in order to determine the mimimal concentration of perfume oil needed to inhibit the growth of fungi *in vitro*.

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The author wishes to thank Magnus, Mabee, and Reynard, Inc., N. Y., for the perfume oils used in this investigation.

Anal.—Calcd. for $C_{20}H_{20}ClN_3O \cdot 2HCl$: C, 56.28; H, 5.20; Cl (ionic), 16.62. Found: C, 55.95; H, 5.36; Cl, 16.26.

4-(6-Chloro-2-methoxy-9-acridylamino)-α-pyrrolidino-o-cresol Dihydrochloride Sesquihydrate.—One-third of the residue from the preparation of II was treated as in that experiment except that 27.8 Gm. (0.1 mole) of 6,9-dichloro-2-methoxyacridine replaced 4,7-dichloroquinoline. The hot reaction mixture was filtered in order to remove insoluble 6-chloro-9-(4-hydroxyanilino)-2-methoxyacridine. Cooling the filtrate gave 30 Gm. (56% yield) of dihydrochloride, m.p. 245–249° dec. It was recrystallized from alcohol-acetone, m.p. 252–253° dec.

Anal.—Calcd. for $C_{25}H_{24}ClN_3O_2 \cdot 2HCl \cdot 1^1/_2H_2O$: C, 56.25; H, 5.47. Found: C, 56.54; H, 5.37.

p-Acetamido-α-hexamethylenimino-o-cresol Monohydrochloride.—A mixture of 15.1 Gm. (0.1 mole) of *p*-acetamidophenol, 9.9 Gm. (0.1 mole) of hexamethylenimine (5), 3 Gm. (0.1 mole) of paraformaldehyde, and 25 ml. of alcohol was heated to boiling for 4 hours. When cooling gave no precipitate, solvent was removed by distillation under reduced pressure. The residual dark oil was dissolved in ether, and excess alcoholic hydrogen chloride added to precipitate a yellow viscous oil. After decantation of the ether, solution in hot isopropyl alcohol and cooling gave 15.3 Gm. (51% yield) of product, m.p. 192–194°. Recrystallization from the same solvent elevated the melting point to 194.5 to 195°.

Anal.—Calcd. for $C_{15}H_{22}N_2O_2 \cdot HCl$: C, 60.29; H, 7.76. Found: C, 60.46; H, 7.96.

4-(7-Chloro-4-quinolylamino)-α-hexamethylenimino-o-cresol (IV).—A mixture of 14.3 Gm. (0.0478 mole) of *p*-acetamido-α-hexamethyleniminо-*o*-cresol monohydrochloride and 20 ml. of 20% hydrochloric acid was heated at reflux temperature for 1 hour. Sodium hydroxide solution was added to the cooled solution until it was barely acidic to Congo red. After the addition of 9.5 Gm. (0.048 mole) of 4,7-dichloroquinoline, the mixture was heated on a steam bath for 2 hours. Then, with cooling, it was made basic to litmus with 10% sodium hydroxide. The precipitated yellow solid was recrystallized twice from benzene to give 3.8 Gm. (21% yield) of IV, m.p. 212–214° dec.

Anal.—Calcd. for $C_{22}H_{24}ClN_3O$: C, 69.19; H, 6.33. Found: C, 69.31; H, 6.35.

It was found that following the procedure for II, which does not entail isolation of a purified acetamido Mannich phenolic base, actually led to a better yield of IV (45%).

4-(7-Chloro-4-quinolylamino)-α-(4-methyl-1-piperazinyl)-o-cresol (V) Trihydrochloride Dihydrate.—Procedure for II was followed; however, the free base was extracted with chloroform instead of being isolated. The extract was washed with water, dried over potassium carbonate, and treated with excess alcoholic hydrogen chloride. The addition of acetone completed precipitation of product. Recrystallization from alcohol gave 28 Gm. (53% yield) of bright yellow crystalline powder, m.p. 240–300° dec. It is very soluble in water.

Anal.—Calcd. for $C_{21}H_{23}ClN_4O \cdot 3HCl \cdot 2H_2O$: C, 47.73; H, 5.72. Found: C, 47.67; H, 5.93.

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Antifungal Properties of Perfume Oils

By JASPER C. MARUZZELLA

The antifungal properties of 30 perfume oils were tested by allowing the organisms to grow in varying concentrations of the oils *in vitro*. Fifteen of the perfume oils inhibited the growth of all test organisms at concentrations ranging from 1:500 to 1:13,000. The remaining 15 oils inhibited some of the test organisms at concentrations up to 1:11,000. Oil of rose no. 81412 otto type, crab apple blossom, and rose briar were found to possess marked antifungal properties. The dermatophytes were extremely susceptible to many of the perfume oils at minute concentrations.

PERFUME OILS were demonstrated to possess remarkable fungicidal properties when studied by the filter paper disk method (1). However, the oils were tested in the undiluted form at which strength they are rarely used in cosmetics and medicaments. Small amounts of perfumery materials are added to toilet articles and dermatological products in an attempt to render the item more fragrant and to mask unpleasant odors. Whether such minute concentrations possess germicidal properties has been suggested (2) but not experimentally established. This investigation was undertaken in order to determine the mimimal concentration of perfume oil needed to inhibit the growth of fungi *in vitro*.

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The author wishes to thank Magnus, Mabee, and Reynard, Inc., N. Y., for the perfume oils used in this investigation.

TABLE I.—INHIBITORY CONCENTRATIONS OF PERFUME OILS ON FUNGI

Oils	Test Organisms					
	A. niger	*S. schenkii*	*P. ovale*	*C. albicans*	*M. gypseum*	*T. mentagropyhtes*
Arabian N	1:1000	1:500	1:1000	1:1000	1:7000	1:6000
Ashton villa no. 6	1:1000	1:1000	0[a]	1:1000	1:6000	1:7000
Bluebell bouquet	1:1000	1:2000	1:500	1:1000	1:3000	1:6000
Bluestone bouquet	1:1000	1:1000	1:1000	1:1000	1:3000	1:4000
Bouquet no. 22	1:500	1:3000	0	0	1:13,000	1:13,000
Bouquet no. 821 lemon odor	1:500	1:1000	1:500	1:500	1:1000	1:3000
Carylopsis no. 602	1:1000	1:1000	0	1:500	1:3000	1:4000
Chypre french type	1:500	1:3000	0	1:500	1:7000	1:11,000
Cologne American	1:500	1:500	0	0	1:3000	1:3000
Cologne "F" European	1:500	1:500	0	1:500	1:1000	1:2000
Colonial bouquet	1:500	1:1000	1:500	1:1000	1:3000	1:6000
Crab apple blossom	1:2000	1:2000	1:1000	1:1000	1:7000	1:7000
Eau de quinine	1:500	1:1000	0	1:500	1:1000	1:1000
Elder buds	1:500	1:1000	0	1:500	1:2000	1:2000
Gardenia, JM	1:1000	1:1000	1:500	1:1000	1:3000	1:6000
Geranium bouquet	1:1000	1:1000	1:500	1:1000	1:2000	1:1000
Jasmine ordinary	1:500	1:1000	0	0	1:6000	1:4000
Jockey club	1:500	1:1000	0	1:500	1:1000	1:4000
Lilas blanc	1:1000	1:2000	1:500	1:500	1:6000	1:7000
Lilas blanc, L. S.	0	1:500	0	0	1:1000	1:500
Lilac water	1:1000	1:1000	1:500	1:1000	1:4000	1:5000
Neutralizer F.A.	1:1000	1:1000	0	0	1:4000	1:4000
Oriental bouquet no. 225	1:1000	1:2000	1:500	1:2000	1:5000	1:5000
Osheana	1:1000	1:3000	0	1:500	1:7000	1:7000
Palma bouquet	1:1000	1:1000	0	1:500	1:3000	1:3000
Pine bouquet supreme	0	0	0	0	1:1000	1:2000
Rose briar	1:2000	1:2000	1:1000	1:1000	1:6000	1:6000
Rose no. 81412 otto type	1:5000	1:11,000	1:1000	1:3000	1:13,000	1:10,000
Rose odorata	1:1000	1:4000	1:1000	1:1000	1:3000	1:6000
Sweetgrass	1:1000	1:1000	1:1000	1:1000	1:3000	1:2000

[a] No inhibition at 1:500 concentration.

MATERIALS AND METHODS

The antifungal properties of 30 perfume oils were determined against growing cultures of *Aspergillus niger* ATCC 6277, *Sporotrichum schenkii* CDC 629, *Pityrosporum ovale* ATCC 12078, *Candida albicans* ATCC 10231, *Microsporum gypseum* CDC A-352, and *Trichophyton mentagrophytes* ATCC 9533. All of the test organisms were cultivated in Sabouraud maltose broth at 22 to 24° and subcultured every 7 days.

Stock solutions of each perfume oil were prepared in 95% ethyl alcohol. Aliquots of the stock solution were added to melted Sabouraud maltose agar, the contents were hand shaken and poured into Petri dishes. One-hundred milliliters of 7-day broth cultures was hand shaken thoroughly to mix spores and mycelial fragments. One-half milliliter of this fungal suspension was used as the inoculum for each dish and 5 ml. was used to inoculate 100-ml. broths used in subsequent experiments. Inhibitory levels were recorded at the end of 4 days at 22 to 24°. All perfume oils were tested in triplicate. Controls were included to demonstrate the lack of inhibition by the alcohol solvent employed. Replicate tests were conducted on different days in an attempt to minimize the day-to-day variation in the test procedure.

RESULTS AND DISCUSSION

The values listed in Table I are the minimal concentrations of oil found to inhibit growth of the test organisms. It may be observed that 15 of the 30 perfume oils produced inhibition of all test organisms at concentrations varying from 1:500 to 1:13,000. The remaining 15 oils prevented the growth of some of the test organisms at concentrations from 1:500 to 1:11,000. Perfume oil of lilas blanc L.S. and pine bouquet supreme showed slight antifungal properties while rose no. 81412 otto type, crab apple blossom, and rose briar were found to possess inhibitory action at high dilutions. Further inspection of the data in Table I seems to indicate that *T. mentagrophytes* and *M. gypseum* were extremely sensitive to all of the oils while *P. ovale* was resistant.

The data presented would tend to support the view that small amounts of certain perfume oils might be used effectively in the control of some dermatophytes. The concentrations used in this study fall well within the limits of their use in scenting toilet articles and medicaments.

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Colorimetric Assay of Amphotericin B

By J. C. CHANG†, A. B. HONIG, A. T. WARREN, and S. LEVINE

AMPHOTERICIN B, like nystatin, is usually assayed by microbiological methods (1, 2). Following the development of the colorimetric assay of nystatin (3), the method was applied to amphotericin B and promising results were obtained.

The procedure of the assay was the same as for nystatin (3), except that the concentration of amphotericin B solution was about 80 mcg./ml. and absorbance measurements were made at 435 mμ. Results of assays of several samples of powder are shown in Table I.

The colorimetric assays show good reproducibility with a standard deviation of about 2%. The results agree well with the microbiological assays which have a standard deviation of about 2.7%.

It was observed that the absorbance of the color extracted into chloroform was lower if the amphotericin B solution was allowed to stand in the presence of sodium hydroxide, indicating that this method, as in the case of nystatin, may also be a stability assay for amphotericin B. Stability experiments and assays of pharmaceutical preparations are now in progress.

TABLE I.—COLORIMETRIC ASSAY OF AMPHOTERICIN B

Sample[a]	Colorimetric Assay[b] (mcg./mg.)	Average (mcg./mg.)	Microbiological Assay[c] (mcg./mg.)
Powder #1	807		
	809		
	772		
	781	792	801
Powder #2	828		
	816		
	820		
	819	821	786
Powder #3	771		
	760		
	765	765	743
Powder #4	877		
	879		
	832	863	864
Powder #5	866		
	886		
	883	878	879
Powder #6	789		
	805	797	802
Powder #7	941		
	920		
	895		
	903	915	908
Powder #8	764		
	796		
	783		
	794		
	804	788	825

[a] Stored at 0° and colorimetric assays performed over a 4-month period. [b] Each value represents an individual assay with an independent sample preparation. [c] Assayed by the turbidimetric method using *Candida tropicalis*.

REFERENCES

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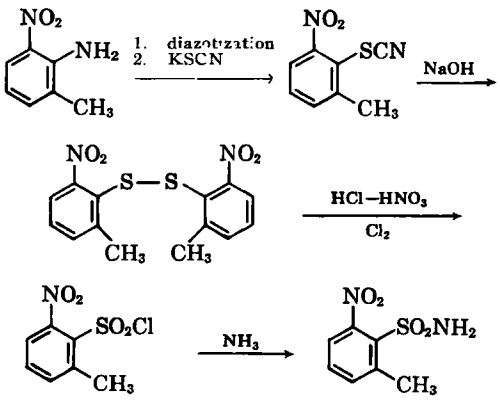
Received August 9, 1962, from E. R. Squibb and Sons, Georges Road, New Brunswick, N. J.
Accepted for publication October 9, 1962.
The authors wish to thank Mr. H. Prager for performing some of the assays.
† Present address: Department of Chemistry, University of California, Los Angeles 24.

Saccharin Derivatives VII. Synthesis of 7-Nitrosaccharin and 7-Aminosaccharin

By GLENN H. HAMOR

RECENT work has shown that alkyl 4-amino-2-sulfamoylbenzoates possessing marked anticonvulsant activity may be prepared by alcoholysis of 6-nitrosaccharin, followed by reduction (1). Therefore, the preparation of 7-nitrosaccharin was undertaken as a starting material for synthesis of further compounds to be used in a structure-activity correlation study of anticonvulsants. In addition 7-aminosaccharin was synthesized in the hope that it, along with 7-nitrosaccharin, might give useful information concerning the relationship of chemical structure to taste (2).

The parent compound, 6-nitro-*o*-toluenesulfonamide (I), was prepared by the method of Szabo (3) according to the series of reactions shown below.



I

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	765	765	743
Powder #4	877		
	879		
	832	863	864
Powder #5	866		
	886		
	883	878	879
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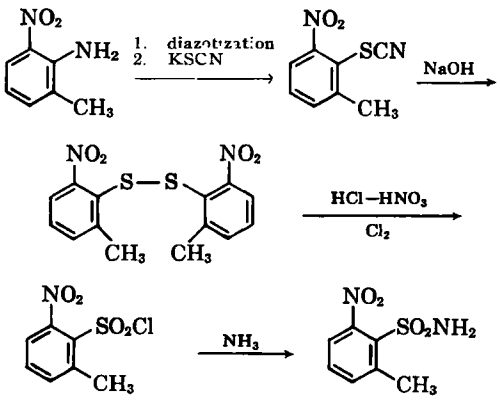

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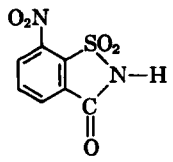
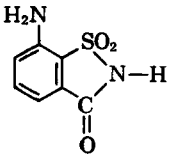
Permanganate oxidation of 6-nitro-*o*-toluenesulfonamide gave 7-nitrosaccharin (II). Reduction of 7-nitrosaccharin by hydrogen with palladium-on-carbon catalyst yielded 7-aminosaccharin (III).



II



III

The 7-nitrosaccharin is essentially tasteless, while 7-aminosaccharin has a sweet taste.

EXPERIMENTAL[1]

7-Nitrosaccharin.[2]—This compound was prepared by alkaline potassium permanganate oxidation of 6-nitro-*o*-toluenesulfonamide by the method used by Noyes (4) to synthesize 6-nitrosaccharin. The 6-nitro-*o*-toluenesulfonamide, m.p. 201–202° [reported m.p. 197–199° (3)], was synthesized according to the series of reactions shown above. Recrystallization of the 7-nitrosaccharin from ethanol gave yields of approx. 25% of yellowish-white crystals, m.p. 262–264° dec. Approx. 30% of unreacted starting sulfonamide was recovered and used in succeeding oxidations.

Anal.[3]—Calcd. for $C_7H_4N_2O_5S$: C, 36.84; H, 1.77. Found: C, 37.02; H, 1.86.

7-Aminosaccharin.—Reduction of 7-nitrosaccharin using hydrogen with palladium-on-carbon catalyst according to standard procedures (5), followed by recrystallization of the product from ethanol gave crystalline 7-aminosaccharin (82%) m.p. 269–270°. Dilute solutions of the compound in ethanol displayed a bluish fluorescence.

Anal.—Calcd. for $C_7H_6N_2O_3S$: C, 42.42; H, 3.12. Found: 42.49; H, 3.09.

[1] Melting points were performed by the capillary tube method and are uncorrected.

[2] *Chem. Abstr.* nomenclature: 7-nitro-1,2-benzisothiazolin-3-one-1,1-dioxide.

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[3] Analyses were performed by Elek Micro Analytical Laboratories, Los Angeles, Calif.

Polytetrafluorethylene Tipped Tablet Punches

By SHELDON SIEGEL†, EDWARD J. HANUS, and JOHN W. CARR

Severe powder sticking to tablet punch surfaces during the production of an effervescent tablet has been overcome by the utilization of punches tipped with polytetrafluorethylene.[1]

The objective of this investigation was to develop an effervescent tablet containing a mixture of sodium isoascorbate and isoascorbic acid which would be rapidly soluble in water and form a solution completely free of turbidity. This tablet was to be used as a source of isoascorbate for meat curing processes to provide a convenient measure for the meat packer who wished to equate his antioxidant requirements to numbers of tablets in preference to units of weight or volume.

The manufacture of an effervescent tablet which yields a clear solution when dissolved in water is not possible when conventional tablet lubricants such as the metallic stearates, mineral oil, etc., are used. For this reason polyethylene glycol 4000 was chosen to act in the dual role of binder and water soluble lubricant. An effervescent tablet blend was prepared and compressed directly on a Stokes model DDS-2 tablet machine into 15-Gm. wafers having a diameter of $1^5/_{16}$ in. The blend had the following composition (1):

Sodium isoascorbate	9.92	Gm./tablet
Isoascorbic acid	2.02	"
Sodium bicarbonate U.S.P. powder	1.06	"
Citric acid anhydrous U.S.P. powder	0.50	"
Polyethylene glycol 4000 (100 mesh)	1.50	"
	15.00	"

When direct compression of this powdered formulation was attempted, severe picking and sticking occurred after compressing relatively few tablets; satisfactory production was impossible because of frequent breakdowns. As an outgrowth of this investigation the utilization of plastics, as components of tablet punches, was studied.

EXPERIMENTAL

Circles of polyethylene film were cut from a plastic bag and bonded to punch faces with a rubber cement. Satisfactory experimental facsimilies of the desired formulation were produced with these punches on a single punch Stokes model F tablet machine. It was noted that the sticking and picking observed in earlier experiments was significantly reduced. Polytetrafluorethylene (2–4) was subsequently investigated because of its inherent self-

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† Present address: Warner-Lambert Research Institute, Morris Plains, N. J.
Grateful appreciation is given to Mr. Edgar H. Miller, Packing Engineering Corp., Cranford, N. J. for his excellent suggestions and assistance.
[1] Marketed as Teflon by E. I. duPont de Nemours and Co., Inc., Wilmington, Del.

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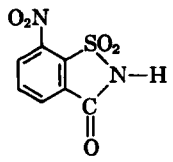

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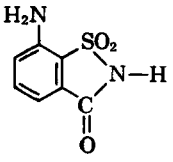

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Anal.[3]—Calcd. for $C_7H_4N_2O_5S$: C, 36.84; H, 1.77. Found: C, 37.02; H, 1.86.

7-Aminosaccharin.—Reduction of 7-nitrosaccharin using hydrogen with palladium-on-carbon catalyst according to standard procedures (5), followed by recrystallization of the product from ethanol gave crystalline 7-aminosaccharin (82%) m.p. 269–270°. Dilute solutions of the compound in ethanol displayed a bluish fluorescence.

Anal.—Calcd. for $C_7H_6N_2O_3S$: C, 42.42; H, 3.12. Found: 42.49; H, 3.09.

[1] Melting points were performed by the capillary tube method and are uncorrected.

[2] *Chem. Abstr.* nomenclature: 7-nitro-1,2-benzisothiazolin-3-one-1,1-dioxide.

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Polytetrafluorethylene Tipped Tablet Punches

By SHELDON SIEGEL†, EDWARD J. HANUS, and JOHN W. CARR

Severe powder sticking to tablet punch surfaces during the production of an effervescent tablet has been overcome by the utilization of punches tipped with polytetrafluorethylene.[1]

The objective of this investigation was to develop an effervescent tablet containing a mixture of sodium isoascorbate and isoascorbic acid which would be rapidly soluble in water and form a solution completely free of turbidity. This tablet was to be used as a source of isoascorbate for meat curing processes to provide a convenient measure for the meat packer who wished to equate his antioxidant requirements to numbers of tablets in preference to units of weight or volume.

The manufacture of an effervescent tablet which yields a clear solution when dissolved in water is not possible when conventional tablet lubricants such as the metallic stearates, mineral oil, etc., are used. For this reason polyethylene glycol 4000 was chosen to act in the dual role of binder and water soluble lubricant. An effervescent tablet blend was prepared and compressed directly on a Stokes model DDS-2 tablet machine into 15-Gm. wafers having a diameter of $1^5/_{16}$ in. The blend had the following composition (1):

Sodium isoascorbate	9.92	Gm./tablet
Isoascorbic acid	2.02	"
Sodium bicarbonate U.S.P. powder	1.06	"
Citric acid anhydrous U.S.P. powder	0.50	"
Polyethylene glycol 4000 (100 mesh)	1.50	"
	15.00	"

When direct compression of this powdered formulation was attempted, severe picking and sticking occurred after compressing relatively few tablets; satisfactory production was impossible because of frequent breakdowns. As an outgrowth of this investigation the utilization of plastics, as components of tablet punches, was studied.

EXPERIMENTAL

Circles of polyethylene film were cut from a plastic bag and bonded to punch faces with a rubber cement. Satisfactory experimental facsimilies of the desired formulation were produced with these punches on a single punch Stokes model F tablet machine. It was noted that the sticking and picking observed in earlier experiments was significantly reduced. Polytetrafluorethylene (2–4) was subsequently investigated because of its inherent self-

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Grateful appreciation is given to Mr. Edgar H. Miller, Packing Engineering Corp., Cranford, N. J. for his excellent suggestions and assistance.
[1] Marketed as Teflon by E. I. duPont de Nemours and Co., Inc., Wilmington, Del.

lubricating property. Polytetrafluorethylene tablet punches were prepared from $^{5}/_{8}$-inch rods and used in conjunction with a $^{5}/_{8}$-inch metal die on the single punch machine. Sticking and picking were almost completely eliminated, but the operation was complicated by the tendency of this plastic to deform under pressure (a phenomenon known as "cold flow"). The punch area that entered the die retained its shape, but the shaft of the punch was deformed and buldged outwards. It was then reasoned that if faces of metal punches were tipped or coated with polytetrafluorethylene, the plastic would be confined within the die cavity during compression, thus preventing deformity. Coating the punch by spraying a thin layer of polytetrafluorethylene was somewhat effective; however, on extended tableting runs the material peeled off. Punches for the single punch machine were subsequently modified by bonding a small polytetrafluorethylene tip to the surface. The tableting operation using the latter punches was quite satisfactory and similar punches were modified and successfully used for the larger production equipment. The use of this device has eliminated much of the difficulty previously experienced with the manufacture of this product.

Several disadvantages were observed during the use of polytetrafluorethylene tipped punches. They are easily damaged and are subject to tip deformity and breakage. Because of the tendency of this plastic to deform under pressure, only flat faced or at most a slightly beveled tablet can be produced with these punches. Monogramming is obviously not practical. Nevertheless, for our specific problem polytetrafluorethylene tipped punches performed satisfactorily under production conditions.

Bonding Polytetrafluorethylene to Punch Surfaces.—Polytetrafluorethylene is not easily bonded to metal surfaces and requires a series of operations to produce a firm seal. An accepted procedure to facilitate bonding is to etch the surface of the plastic with molten sodium and cement it under high pressure to the metal surface by means of an epoxy resin cement. Tipped punches prepared in this manner were satisfactory but were subject to cleavage of the plastic component from the metal surface after a period of use. Modification of the bonding technique by first spraying the punch surface with a layer of pure molybdenum in order to produce a rough surface for greater plastic adherence significantly reduced this breakage (5).

SUMMARY

A method has been developed for preparing by direct compression a large effervescent tablet containing a mixture of sodium isoascorbate and isoascorbic acid which dissolves to form a solution free of turbidity or haze. The use of tablet punches tipped with polytetrafluorethylene has successfully circumvented the major problem of sticking and picking associated with the manufacture of this product.

The utilization of polytetrafluorethylene tipped punches may find application in other tablet formulations exhibiting similar difficulties. In addition, preliminary experimentation with this device has been successful in reducing lubricant levels in certain tablet blends and further investigation in this area is warranted.

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Communications

Inactive Prednisone Tablets U.S.P. XVI

Sir:

We wish to report a case in which prednisone tablets meeting U.S.P. XVI specifications were found to be clinically inactive *in vivo* and some *in vitro* test results which suggest a possible reason for their inactivity.

A 25-year-old white married female of Mediterranean ancestry has been under the care of one of us (FAC) for approximately 5 years. Her clinical diagnosis was familial Mediterranean fever with repeated episodes of clinical peritonitis confirmed by laboratory studies and previous surgical exploration. The prompt use of oral prednisone in amounts of 20 mg. in a 24-hour period for the first 2 or 3 days would promptly abort the clinical symptoms, the laboratory findings of leukocytosis, etc., changing only slightly. The patient's prescriptions had been written with the generic name "prednisone." On one occasion, after 72 hours of 5 mg. four times a day, the patient had no clinical effects from the medication. It was discovered at that time that a different "brand" of prednisone had been dispensed than that previously used. The patient was immediately transferred to the brand of prednisone used previously and again within 24 hours there was almost complete resolution of the clinical syndrome.

The manufacturer's reassay of tablets, from the same lot as the tablets which were ineffective in treatment of the condition described, indicated that they contained essentially all of their labeled content. Also, the tablets passed the

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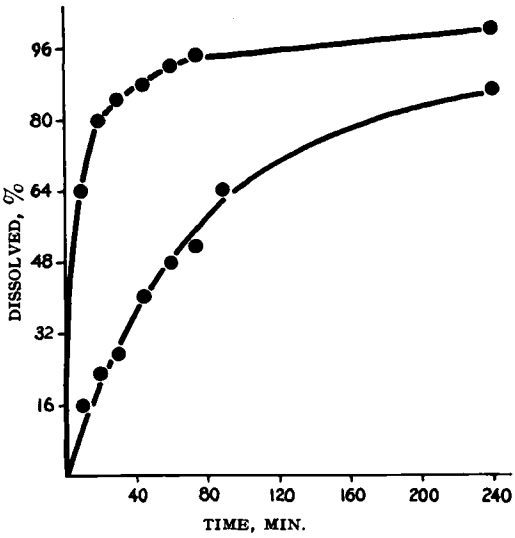

Fig. 1.—Cumulative per cent prednisone tablets dissolved *vs.* time from clinically active (upper curve) and inactive (lower curve) tablets of this substance.

U.S.P. XVI disintegration time test well within prescribed limits.

A small number of tablets were obtained from the manufacturer for a more detailed examination of their physical properties. The U.S.P. XVI disintegration time test was repeated in one of these laboratories and all the tablets had disintegrated within 6 minutes. It was observed, however, that large particles of the tablets remained on the bottom of the test beaker used in the procedure. In addition, during the conduction of the test it appeared that the disk used in the apparatus formed the large particles and subsequently forced them through the screen at the bottom of the testing tubes. One tablet was subjected to the disintegration time test without the disks and at 25°. The tablet had not disintegrated within 1 hour but had disintegrated within 2 hours.

In order to study the problem further, the remaining tablets were examined by means of a dissolution rate test. For comparison, tablets of the brand (which also disintegrated in less than 6 minutes in the U.S.P. XVI test) which gave a satisfactory clinical response were also subjected to the same test. The solution rate test conditions and apparatus used were those described by Levy and Hayes (1). The dissolution media were 500 ml. of water maintained at 37° and aliquots of it were assayed spectrophotometrically at 240 mμ for drug content at various times.

Figure 1 shows cumulative per cent of drug released from the two brands of tablets tested.

TABLE I.—SUMMARY OF DISINTEGRATION AND SOLUTION RATE DATA

Prednisone Tablets	U.S.P. XVI Disintegration Time (with disk)	Disintegration Time (without disks)	Av. time for 50% of Drug to Dissolve, ± S. D. Dev.
Active lot	<6 min.	<6 min.	4.3 ± 1.3 min.
Inactive lot	<6 min.	1–2 hr.	100 ± 53 min.

It is apparent from this figure that the tablets which were clinically ineffective dissolved much more slowly than the effective ones. The effective tablets released 50% of their content more than 20 times as rapidly as the ineffective ones.

Each point on the curve of Fig. 1 showing dissolution rate behavior of the clinically effective tablets is the average of six determinations. In the case of the ineffective brand, each point is the mean of three determinations. It was observed that the release of prednisone from the ineffective tablets was much more erratic in regard to tablet-to-tablet variation than from the effective brand of tablets. The various data obtained in this study are summarized in Table I.

This study provides additional evidence to previously published work (1–8) suggesting that the U.S.P. disintegration time test should be re-evaluated as a method to predict correctly physiological availability *in vivo*. The fact that a dissolution rate test gave results that appeared to be in qualitative agreement with clinical experience does not mean that the particular test used will necessarily be applicable to predict physiological availability from tablets of other drugs.

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Rapid Detection of Excess Glucose in Coloring Syrups Used in Tablet Production

Sir:

When more than 2% glucose is present in the coloring syrup applied in tablet coating, the resulting tablets may be inelegant and exhibit a blotchy, wrinkled, or leathery surface. Such unmarketable tablets represent a substantial loss in material, labor, and time. This loss can be avoided if the offending batches of coloring syrup can be detected before they are used on the tablets.

We have found a simple procedure using a dip-and-read stick test, Clinistix,[1] to identify batches of coloring syrup that contain excess glucose. The stick test contains the enzyme glucose oxidase and so is specific for glucose. To use the test, the reactive area of the stick is dipped in a solution and removed. If glucose is present in the solution, the reactive area (originally red) turns purple. The purple can be compared with the color chart provided to determine if a little or a lot of glucose is present. The test was originally designed to detect glucose in urine.

To identify unsatisfactory batches of coloring syrup, we use a different procedure from that suggested by the manufacturer for the detection of glucose in urine. We prepare two dilutions of the coloring syrup, one of 2 drops in 10 ml. of water (1:110 dilution) and one of 5 drops in 10 ml. of water (1:44 dilution). (Under the conditions of this study there were 22 drops of coloring syrup in 1 ml.) One stick is dipped in each of these dilutions, removed, and the color allowed to develop for 30 seconds. The stick dipped in the 2-drop dilution is compared with the light color on the color chart and the stick dipped in the 5-drop dilution is compared with the medium color on the color chart. If either of the sticks is darker than the color it is compared with, the batch of syrup is discarded as containing too much glucose. Our experiments indicate that sticks dipped in the above dilutions of clear syrup (without dye) containing more than 2% glucose will give colors darker than the control colors on the chart. Sticks dipped in the same dilutions of clear syrup containing less than 2% glucose will give colors lighter than the control colors on the chart.

We have also carried out the stick procedure on some syrups containing dyes. We found the procedure as described satisfactory with syrups containing FD&C Yellow No. 5, FD&C Red No. 4, and an orange made by combining these two dyes. The procedure as described was not satisfactory with syrups containing FD&C Blue No. 1 and Hercules Mint Green. In both of these colored syrups, sticks dipped in dilutions of syrup which originally contained 1% glucose gave colors darker than the control colors on the chart. It is clear that the procedure would have to be modified to detect excess glucose in syrups of these colors. It is our opinion that for any particular operation the investigator should develop his own procedure with the stick test. The material presented here is only meant to illustrate that a practical system for detecting batches of coloring syrup containing excess glucose can be developed simply.

Our coating operation has been helped by the stick test control procedure. From the time we first encountered the excess glucose problem until we began testing all batches of coloring syrup, 21% of the tablets were rejected for discoloration resulting from excess glucose in the coloring syrup. Since we began testing all batches, no tablets have been rejected for discoloration related to the excess glucose.

When the cause of irregular coloring on tablets is obscure, the stick test can be used to determine if excess glucose in the coloring syrup used was a factor. To use the test for this purpose, three of the irregularly colored tablets are placed in 30 ml. of water in a small beaker. The tablets are left in the water until their color has been washed off, then are quickly removed. If the tablets are left in the water longer, sugar from subcoatings is washed off and the test results are not valid. A stick is dipped in the water in which the tablets were washed. At 30 seconds the color on the stick is compared with the color chart. If excess glucose is present in the tablet coloring, the stick will give a reaction darker than the medium color on the chart. This procedure is satisfactory for our coating operation; others who might want to use the test for this purpose would probably have to establish their own procedure empirically.

The undesirable glucose apparently is produced in the coloring syrup under normal production conditions. To determine how glucose might be produced, a simple experiment was carried out. One sample of freshly prepared coloring syrup was refrigerated for 3 days and a second sample of the same syrup was put in a small flask and heated in an oven at about 70° for 3 days.

[1] Marketed by the Ames Co. Inc., Elkhart, Ind.

The refrigerated sample showed no buildup of glucose; the heated sample developed about 2% glucose. In another experiment, one sample of freshly prepared coloring syrup was allowed to stand for 3 days at room temperature; a second sample of the same syrup was heated for 3 days at 70° in steam kettles used in preparing the syrup. The syrup allowed to stand at room temperature contained about 1% glucose after the 3 days. The heated sample contained about 8% glucose (higher than the sample heated in the oven, probably because the inner surface of the steam kettle has a higher temperature than the 70° of the oven). It appears that glucose can be produced by the hydrolysis of sucrose in coloring syrups that are heated for some time.

Excess glucose probably causes the unacceptable tablet surface by retarding the drying of the coating material. The moist tablets tend to be sticky and adhere to one another and to the pan. In this state the surface is subject to tiny "pulls," eruptions, and general surface disfigurement as a result of repeated sticky collisions. Coloring syrups with excess glucose are much more likely to result in disfigured tablets with inexperienced coaters than with experienced coaters who can recognize the trouble and modify their procedure to allow proper drying of the tablets.

The stick control procedure has been very useful in our coating operation. The stick test is simple enough that modifications in procedure are practical. We think the test might be useful to others for whom the determination of glucose in some phase of tablet manufacture is important.

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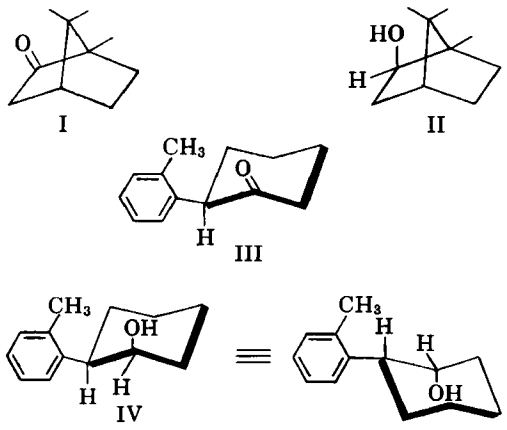
Asymmetric Synthesis in the Stereoselective Reduction of 2-*o*-Tolylcyclohexanone by Isobornyloxymagnesium Bromide

Sir:

Subsequent to the synthesis of optically active mandelic acid by the reduction of phenylglyoxylic acid with isobornyloxymagnesium bromide by Vavon and Antonini (1) other investigators (2–4) have used the same reducing agent for the asymmetric synthesis of optically active deuterated primary alcohols of known absolute configuration from symmetrical aldehydes and deuterio-isobornyloxymagnesium bromide or from isobornyloxymagnesium bromide and symmetrical 1-deuterio-aldehydes.

We wish to report the synthesis of optically active *cis*-2-*o*-tolylcyclohexanol by the reduction of racemic 2-*o*-tolylcyclohexanone with isobornyloxymagnesium bromide. The high degree of stereoselectivity of the reaction is indicated from the fact that the 2-*o*-tolylcyclohexanol obtained consist of about 92% *cis*-2-*o*-tolylcyclohexanol, $[\alpha]_D^{24} = -2.9$ (0.1 Gm./ml. in ethanol) (optical purity unknown), and only about 8% of the *trans* isomer. The *trans* isomer was not recovered in sufficient amount for optical rotation measurements. Nonspecific reduction of racemic 2-*o*-tolylcyclohexanone would of course yield the four optical isomers of 2-*o*-tolylcyclohexanol in equal quantities. Since *cis* alcohol is formed preferentially, the energy of activation for the transfer of the hydride ion to the carbonyl carbon of 2-*o*-tolylcyclohexanone is lower when the transfer leads to an equatorial C—H bond. Because the resulting *cis* alcohol has optical activity, the energy of activation is lower for the formation of the *cis* alcohol from one enantiomorph of 2-*o*-tolylcyclohexanone than from the other. Molecular models indicate that the least hindered of the four possible transition states is the one resulting from the interaction of II and that enantiomorph of 2-*o*-tolylcyclohexanone shown by structure III to give product IV.



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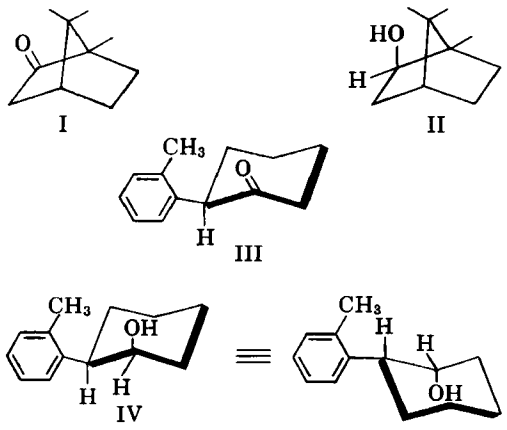

Structure IV is suggested as the absolute con-

figuration of the optically active *cis*-2-*o*-tolylcyclohexanol obtained from the stereoselective reduction. This is consistent with the mechanism for stereoselective reduction by isobornyloxymagnesium bromide proposed by Streitwieser and co-workers (3) based on structure I being the absolute configuration of (+)camphor (3, 5) from which the isoborneol was prepared.

The isoborneol prepared by lithium aluminum hydride reduction of (+)camphor according to the method of Noyce and Denney (6) had $[\alpha]_D^{25} = -23.0°$ (*c* 5 in ethanol) compared to $-26.0°$ reported by these authors for a product which was reported contaminated with about 10% borneol (6). It has been shown (1, 3) that the presence of this amount of borneol does not affect the course of reduction by isobornyloxymagnesium bromide because borneol reacts so much slower under the same conditions. We have purified the isoborneol by elution chromatography on alumina to give $[\alpha]_D^{25} = -30.0°$ (*c* 5 in ethanol) (reported value (7) for pure isoborneol, 8% in ethanol, is $[\alpha]_D^{19} = -33.5°$) and have found no apparent difference in the stereospecificity of the reduction by the magnesium bromide salt of the purified isoborneol compared to that obtained from isoborneol contaminated with some borneol. A solution of racemic 2-*o*-tolylcyclohexanone in anhydrous benzene was added to a benzene solution of isobornyloxymagnesium bromide, prepared by the method of Streitwieser (3), and the mixture was heated to reflux. The progress of the reaction was followed by periodic work-up of small portions of the reaction mixture and analysis of the product by gas chromatography in comparison to the known *cis*- and *trans*-2-*o*-tolylcyclohexanols (8). Two conditions, referred to as *Methods A* and *B*, were used. In *Method A* slightly over two equivalents of the racemic ketone were used and the refluxing was carried on until practically all the isoborneol was depleted (about 45 hours of refluxing). In *Method B* two equivalents of isobornyloxymagnesium bromide were used and the reaction carried out until the ketone was practically depleted (about 60 hours of refluxing). The 2-*o*-tolylcyclohexanol obtained by *Method B* consisted of about 92% *cis* isomer and 8% *trans*. This ratio appeared to remain constant during the course of the reactions. The product of *Method A* consisted of about 91.5% *cis* after 23 hours of refluxing and about 89% *cis* and 11% *trans* at the end of 45 hours of refluxing. The *cis* isomer was recovered by preparative gas chromatography, using a 5-ft. column packed with 18% Carbowax 20M on acid-washed Chromosorb W. The *cis* product of reaction *A* had $[\alpha]_D^{24} = -2.88°$ and that from reaction *B* $-2.95°$. Resolution of *cis*-2-*o*-tolylcyclohexanol is in progress in order to establish the optical purity of the product of the asymmetric synthesis. The unreacted ketone recovered from reaction *A* was not optically active. This fact and the fact that complete reduction of the racemic ketone yielded optically active *cis* alcohol in reaction *B* indicates isomerization of the ketone through enolization. If isomerization did not occur, the remaining ketone in reaction *A* would be richer in the mirror image of structure III.

In a (Meerwein-Pondorff)-Oppenauer reaction of the type used, many species can be in equilibrium. The initial reaction between the isobornyloxymagnesium bromide and the 2-*o*-tolylcyclohexanone could be reversible, and the magnesium bromide salts of the 2-*o*-tolylcyclohexanols could possibly equilibrate with unreacted 2-*o*-tolylcyclohexanone. The apparent constant ratio of *cis* and *trans* products in reaction *B* and the apparent increase in *trans* isomer, with time, in reaction *A* suggest a tendency toward equilibrium between products and unreacted ketone. It is of interest that from molecular models and Streitwieser's mechanism (3) the reaction of the optically active product (salt of IV) with 2-*o*-tolylcyclohexanone is predicted to be faster with the enantiomorph shown by structure III, and that the most rapid reaction will yield IV as the product. This possibility is being investigated further.

Steric control of the reduction of certain cyclanones by diisopinocamphenylborane has been reported by Brown and Bigley (9).

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Per Cent Absorbed Time Plots Derived from Blood Level and/or Urinary Excretion Data

Sir:

Dominguez and Pomerene (1) derived an equation, which had a form similar to Eq. 1, for calculating the rate of absorption as a function of time from blood level data

$$\frac{dA}{dt} = V\left(\frac{dC}{dt} + K.C\right) \qquad \text{(Eq. 1)}$$

Here, dA/dt is the rate of absorption or the rate of appearance of the substance in the blood, V is the apparent volume of distribution, C is the blood level in concentration units at time t, dC/dt is the slope of the blood level time curve at time t, and K is the first-order rate constant for loss of drug from the volume of distribution.

The assumptions behind Eq. 1 are: (*a*) equilibrium between drug in blood and other fluids of distribution is maintained; (*b*) V and K are constants, independent of time, over the time interval Eq. 1 is applied; and (*c*) the compound measured in the blood is the same compound as that absorbed (*i.e.*, A, in weight units, and C, in concentration units, refer to the same compound).

Nelson (2) derived Eq. 2 which may be used for calculating the rate of absorption as a function of time from urinary excretion data

$$\frac{dA}{dt} = \frac{1}{f}\left(\frac{1}{K}\cdot\frac{d^2A_e}{dt^2} + \frac{dA_e}{dt}\right) \qquad \text{(Eq. 2)}$$

Here, f is the fraction of drug reaching the circulation which is excreted unchanged in the urine, d^2A_e/dt^2 and dA_e/dt are the second and first derivatives, respectively, of a plot of cumulative amount of unchanged drug in the urine against time, and dA/dt and K have the same meaning as above. dA_e/dt is the rate of excretion at the time when the cumulative amount excreted is A_e. Theoretically, excretion rate is directly proportional to blood level (3); hence, substitution of $(1/V.K.f).(dA_e/dt)$ for C in Eq. 1 yields Eq. 2.

The assumptions behind Eq. 2 are: (*a*) equilibrium between drug in blood and other fluids of distribution is maintained; (*b*) f, K, and the renal clearance ($f.K.V_d$) are constants, independent of time, over the time interval Eq. 2 is applied; (*c*) $dA_e/dt = f.dA_e'/dt$ where dA_e'/dt is the rate of elimination of the drug from the volume of distribution by all processes; and (*d*) the compound measured in the urine is the same compound as that absorbed (*i.e.*, both A and A_e are weight units of the same compound).

The difficulties experienced in using Eqs. 1 and 2 are: (*a*) values of V and f are only determined, even with reasonable accuracy, by means of intravenous studies; (*b*) the second derivatives, d^2A_e/dt^2, are difficult to obtain with accuracy.

We wish to report that per cent absorbed–time plots may be derived directly from suitable blood level and/or urinary excretion data obtained following oral or parenteral administration of a drug without knowing the values of V, f, or d^2A_e/dt^2.

Integration of Eq. 1 between the limits $t = o$ and $t = T$ yields Eq. 3

$$A_T = V\left(C_T + K\int_{t=o}^{t=T} C.dt\right) \qquad \text{(Eq. 3)}$$

Here, A_T is the amount of drug absorbed from time of administration to time T, C_T is the blood level at time T, and the integral is the area under the blood level time curve between time zero and time T. Equation 3 has been previously published (4).

Integration of Eq. 2 between the limits $t = o$ and $t = T$ yields Eq. 4

$$A_T = \frac{1}{f}\left(\frac{1}{K}\cdot\frac{dA_e}{dt} + A_e\right) \qquad \text{(Eq. 4)}$$

Rearrangement of Eqs. 3 and 4 yields Eqs. 5 and 6

$$\frac{A_T}{V} = C_T + K.\int_{t=o}^{t=T} C.dt \qquad \text{(Eq. 5)}$$

$$f.A_T = \frac{1}{K}\cdot\frac{dA_e}{dt} + A_e \qquad \text{(Eq. 6)}$$

Equations 5 and 6 are those suitable to prepare per cent absorbed–time plots from blood level and urinary excretion data, respectively. The method using blood level data is as follows. Successive values of the righthand side of Eq. 5 are calculated from the time of administration ($t = o$) to some time after the peak in the blood level time plot (the areas may be estimated with the trapezoidal rule). The values progressively increase, then reach a maximum or asymptotic value. When the individual values are expressed as percentages of the maximum or asymptotic value, the results are per cent absorbed values to various times T. The method using a cumulative amount excreted–time curve is as follows. For the initial part of the plot, values of $\Delta A_e/\Delta t$ and the amount excreted at the midpoint of the interval, A_e, are calculated. The operations dictated by Eq. 6 are then performed to yield values of $f.A_T$. The values pro-

gressively increase, then reach a maximum or asymptotic value. When the individual values are expressed as percentages of the maximum or asymptotic value, the results are per cent absorbed values to various times. Theoretically, the maximum or asymptotic value of the right-hand side of Eq. 5 is $K.\int_{t=o}^{t=\infty} C.dt$ and of Eq. 6 is $(A_e)_\infty$, where $\int_{t=o}^{t=\infty} C.dt$ is the area under the blood level time plot from $t = o$ to $t = \infty$, and $(A_e)_\infty$ is the amount of unchanged drug excreted in the urine in infinite time. The methods are independent of the values of V and f since these values cancel out when the percentages are calculated. The per cent absorbed value so calculated is the cumulative amount absorbed to time T expressed as a percentage of the total amount of drug which is absorbed (not as a percentage of the dose).

When the cumulative percentages absorbed are plotted against time, the resulting plots may contain linear segments; the slope of such a linear segment is the absorption rate in per cent/hour. If the plot is curved, or contains curved and linear segments, it may often be resolved to yield the components of the rate $d\%A_T/dt$. In this way the model which applies to a particular set of blood level or urinary excretion data can be determined accurately. The method may also be applied to problems in chemical kinetics involving consecutive and/or simultaneous first-order and zero-order reactions.

The process of obtaining the per cent absorbed–time plots from blood level data has been automated in two ways. In the first method, the blood level data are plotted on suitable graph paper and the points joined with a smooth line using a special conducting ink. Using a curve-follower, an analog computer was programmed to perform the operations dictated by Eq. 5 and to plot the result on another piece of graph paper. The asymptotic value is estimated from the plot, the values of A_T/V are obtained from the plot, and then expressed as a percentage of the asymptotic value. In the second method, the blood level–time values are fed to a suitably programmed digital computer. The computer calculates the cumulative areas, using the trapezoidal rule, then performs the necessary operations shown in Eq. 5. The print-out contains the values T, C_T, $\int_{t=o}^{t=T} C.dt$, and $\left[C_T + K.\int_{t=o}^{t=T} C.dt\right]$. The latter are then expressed as percentages of the asymptotic or maximum value of the same function to yield the cumulative per cent absorbed values.

Per cent absorbed–time plots derived from *in vivo* data are the optimum data to correlate with per cent released time plots derived from *in vitro* testing.

Future publications will illustrate applications of these methods in detail.

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Book Notices

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The Editor comments

FISCAL RESPONSIBILITY IS ESSENTIAL

In recent months a sister organization in the field of the biological sciences found itself in very serious difficulty because of certain fiscal irregularities which had occurred in its administration of sizeable grants awarded by the National Science Foundation. The organization involved has subsequently taken heroic measures to raise sufficient funds to reimburse the NSF for the entire sum of over a quarter of a million dollars which allegedly has been misused.

Almost simultaneously, Congressional investigators have brought to light several incidents in which funds provided under the National Institutes of Health grants program have been employed for the purchase of unauthorized equipment or in which budgets have been adjusted internally to allocate disproportionately for administrative or overhead costs.

Several features common to the cases involving both the NSF grants and the NIH grants appear to deserve some comment.

In the first place, in none of these disclosures was there any evidence of purely personal gain involved in the misdirection of funds; on the contrary, all evidence brought to light indicated that the irregularities were entirely motivated by unrestrained scientific zeal. Those entrusted with administering the grants made certain improper compromises in an effort to achieve more rapidly the desired goals of the specific study, or to expedite other phases of their general program of research activities.

Secondly, the overall effect of these disclosures has been to raise doubts in the minds of both Congress and the public as to the fiscal awareness and sense of responsibility which might be expected either from individual scientists or the scientific community. On this point Dr. James D. Ebert, director of the embryology department of the Carnegie Institution of Washington, has stated: *"Our lofty ideals, our high purpose in the laboratory and classroom, have not always been carried over in our day to day business practice; we have been more concerned with the advancement of our own fields than with safeguarding the public interest."*

Clearly, it is the individual responsibility of each grant recipient to exercise all diligence in the management of the funds entrusted to him by his fellow citizens through government agencies or private foundations. Scientists need to recognize that in financial matters attentiveness to details and accuracy must be exercised comparable to that employed in reporting on research experiments.

Edward G. Feldmann

The postal zone number for the JOURNAL OF PHARMACEUTICAL SCIENCES, as well as all other mail directed to the AMERICAN PHARMACEUTICAL ASSOCIATION, will be changed from "7" to "37," effective July 1, 1963.

JOURNAL OF Pharmaceutical Sciences

July 1963 volume 52, number 7

Review Article

Pharmaceutical Sciences—1962. Part I

A Literature Review

By WAYNE McKEEHAN

CONTENTS

[Contents for Part II will accompany the concluding part of Dr. McKeehan's Review Article next month.]

THIS REVIEW covers the literature of pharmaceutical sciences reported in readily available journals or abstracted in the Pharmaceuticals or Pharmacodynamics sections of *Chemical Abstracts* in 1962. It is intended to be reasonably comprehensive; but the large volume of literature published last year prevented the inclusion of some papers, and others may have been inadvertently overlooked. The general aim is to aid the reader in becoming better acquainted with recent developments by presenting a representative sample of papers appearing in various areas of pharmaceutical science.

Some of the literature related to pharmaceutical sciences has been adequately reviewed elsewhere on an annual basis and is omitted. For such associated papers in analytical chemistry, antibiotics, bacteriology, biochemistry, biology, cancer, medicine, medicinal chemistry, microbiology, organic chemistry, pharmacology, physical chemistry, physiology, and plant physiology, the reader is referred to reviews in those areas. The *Advances in . . .* series, the *Annual Reviews of . . .* series, and the *Progress in . . .* series are particularly pertinent.

For the purpose of this review, the selected references have been divided into eight sections. These sections have been subdivided further, where necessary, in order to group related papers. The divisions were established arbitrarily and have no special significance other than to facilitate organization.

GENERAL PHARMACY

Included in this rather broad area are various portions of the literature which did not blend easily into the other divisions. Breunig and King discussed problems and effectiveness of modern statistical methods in acceptance sampling of finished pharmaceutical products (1). Investigations of the influence of seaweed products on the absorption of drugs were reported (2). Publications have appeared concerning the compatibility and manipulation of guar gum (3) and the incompatibilities of various compounds in pharmaceutical preparations (4, 5). Suspension sampling, studied by Anderson and Shaw, yielded the most consistent results when samples were obtained by weight (6). The influence of pH on surface charactertistics of silica gel in aqueous solutions was investigated (7). Brooks, *et al.*, cataloged solid pharmaceutical dosage forms for use in forensic toxicology (8), and Wagner suggested the use of a manually sorted punched-card system for pharmaceutical literature (9). Other writers discussed physical and physiological aspects of collyria (10) and the organization and economics of drug research (11).

Preservatives.—Bailey discussed and classified various antioxidants according to their mechanism of action (12). Physicochemical aspects of preservative selection for emulsions (13) and a manometric method for evaluation of preservatives (14) were reported. In the latter paper, Wailes suggested using the Warburg apparatus for screening potential preservative agents. Factors influencing the stability of antibacterial preservatives in parenteral solutions were explored by Lachman, *et al.* (15). The interaction of preservatives with certain macromolecules was also studied (16). Dimethoxane, a new preservative, retained its activity in the presence of nonionic emulsifiers (17).

Several papers concerning the use of hydroxybenzoate esters as preservatives were published in 1962. The role of alkyl *p*-hydroxybenzoate esters as pharmaceutical preservatives was reviewed (18). Matsumoto and Aoki studied the inactivation of *p*-hydroxybenzoic acid esters in the presence of surfactants (19, 20). Activity appeared to be dependent on the amount of material in the aqueous phase outside of the surfactant micelle. Another monograph reported research on the fungistatic activity of methyl and propyl hydroxybenzoates (21), and mixed parabens were used by Nuppenau for the preservation of hexachlorophene cream (22).

Humberstone found that chlorhexidine diacetate was not a suitable preservative for eyedrops in the presence of sulfate ions (23). Potassium metabisulfite had no inhibiting influence on the cleavage of Novocaine in solution (24). The antioxidant action of phenols was explored by McGowan and Powell (25). In a study of the preservation and degradation of adrenaline solutions, sodium pyrosulfite was added as a stabilizer to solutions of various adrenaline salts (26). A preliminary report was published on the effect of a quaternary ammonium compound on polyvinyl chloride (27).

Flavoring.—Pharmaceutical flavoring and its importance to the patient and the physician were reviewed by Moorish (28). The psychology, physiology, and physicochemical aspects of flavor and taste have been discussed (29, 30). A trained taste panel was used in studying the effectiveness of monosodium glutamate for masking bitter taste (31). Mauer continued his series on flavors and per-

Received from the Pharmaceutical Research Department of The Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind.

fumes from various flowers and leaves (32, 33); and many publications appeared on the isolation, identification, and pharmaceutical applications of essential oils (34–39). The utilization and evaluation of natural and synthetic sweetening agents were reported (40–42). Vasic has discussed the perfuming of various types of cosmetic formulations (43).

Adjuvants.—A study of the influence of solvent composition on the activity of various bacteriostats and bactericides was reported (44). The current status of certified colors has been summarized (45). Swartz and Cooper reviewed the chemistry, applications, and properties of various colorants (46), and Hamilton discussed the selection of colors and their effects (47). The properties and uses of linalyl esters as perfumes were investigated (48). Reviews were published on the utility of sorbitol in pharmaceutical liquids (49) and the use of sulfur in pharmacy and cosmetics (50). Pail and Todd discussed functions and formulations of three silicones (51), and Secard dislcosed some new pharmaceutical uses of Carbopol (52). Surfactant applications in pharmacy and cosmetics have been reviewed (53–55). Lower compiled a comprehensive listing of British surface-active agents (56–63). In studying the germicidal activity of hexachlorophene, Banks and Huyck investigated the effects of some wetting agents (64). Russell and Hoch described processes for the preparation of clear detergent solutions containing lanolin oil (65). A polyethylene oxide ether–fatty acid alkanolamide mixture was used to solubilize the lanolin oil.

Stability.—As indicated by the references which follow, the pharmaceutical scientist has shown considerable interest in the development of better methods for evaluating product stability. The influence of light on pharmaceutical formulations has been reported (66). In a short communication, Ramwell and Shaw suggested that aqueous solutions of picrotoxin are stable to light if stored in acid-washed glassware (67). Several investigators reported their work on the stability of tetracycline and its various derivatives (68–70), and the stability of nystatin in dry preparations was demonstrated (71). Physicochemical studies on the stability of various penicillin derivatives have been published (72–74). Citri and Garber concluded that the side chain of a penicillin may confer resistance to hydrolysis by penicillinase (75). A study of the stability of penicillin G sodium in the presence of nonionic surface-active substances was made (76). The stability of *p*-aminosalicylic acid in aqueous solution has been studied (77, 78). Various buffer salts were used by Tanaka and Takino to examine the stability of *p*-aminosalicylic acid and its salts (79). Research on stability and stabilization of acetylsalicylic acid in aqueous solutions was reported (80, 81). Hydrolysis of the undissociated acetylsalicylic acid molecule was suppressed by anionic, cationic, and nonionic surfactants. Decomposition of amethocaine hydrochloride solutions was accelerated by heat and high pH (82). Pantocaine solutions showed no detectable decomposition immediately after sterilization as measured by the diazo reaction, but solutions at several pH values gave a positive test after storage (83). Samuelsson investigated the stability of digitoxin in galenical preparations (84). The stabilities of solutions containing papaverine hydrochloride (85) and some reputedly unstable vegetable drugs (86) were examined.

The stabilization of arsenic solutions (87) and solutions of ferrous gluconate (88) has been studied. Bamann, *et al.*, considered the decomposition of phosphoric acid esters by metal ions at acid pH (89). An experimental method for studying the stability of cortisone and hydrocortisone was published by Takubo, *et al.* (90). Sodium metabisulfite was recommended as an antioxidant for solutions of isoprenaline (91). Most of the racemization of *l*-noradrenaline hydrochloride solutions was found to occur during the first 30 days (92). Nielsen found that poly(vinylpyrrolidinone) prolonged the effect of vasopressin and oxytocin but decreased the thermal stability of their solutions (93). Reports have appeared on the stabilization of solutions of thioproperazine (94) and chlorpromazine hydrochloride (95). Nicotinamide protected the latter from discoloration by light. The combined use of polyhydric alcohols and calcium acetate produced a stable bacterial alkaline proteinase solution (96). In another study, calcium was used to stabilize trypsin in a polyethylene glycol ointment base (97). Nogami and Awazu investigated the stabilization of methantheline bromide in aqueous solution by surface-active agents (98). Other investigations were made on the effect of surfactants on the stability of some pharmaceuticals (99) and the effect of ultrasonic waves on the stability of surfactants, sulfonamides, and *p*-aminobenzoic acid (100). Accelerated aging was used in the evaluation of pharmaceutical fat vehicles (101). Stability studies on buffer solutions (102) and pepsin elixir (103) have been reported. This elixir be-

gan to lose its pepsin activity after storage for 5 months. The influence of additives on the stability of glucose solutions was investigated by Parke (104).

Stability Kinetics.—Chemical kinetics has become a very useful tool in studying the stability of different formulations and in the prediction of shelf life. Sesaki, *et al.*, explored the application of chemical kinetics to the stability of chemicals (105). A method of plotting kinetic data to determine reaction order and specific rate constant has been suggested; evaluation of data up to 99% conversion was possible (106). Hochanadel described a simple device for determining slopes using the glass-rod method (107). In testing the stability of pharmaceutical preparations in a homogeneous liquid phase, the van't Hoff-Arrhenius relation was found useful (108). The kinetics of degradation of epinephrine in solution by molecular oxygen was studied by Sokoloski and Higuchi (109). An extensive study was conducted by Riegelman and Fischer on the stabilization of epinephrine—chelation of the catechol nucleus with boric acid gave marked stabilization (110, 111). Rates of degradation, half-life, and the effect of pH on the degradation of potassium phenethicillin in aqueous solution were discussed (112). The kinetics of degradation of glutethimide (113) and the stability of chloral hydrate solutions (114, 115) have been investigated. Studies on the aging of pharmaceutical preparations were reported by Inami, *et al.* (116, 117). The stability of pyridine-2-aldoxime methiodide in aqueous solutions was evaluated at different temperatures and pH values, and general equations for its half-life were derived (118). Chemical kinetics was used by Heimlich to study glucose degradation in acid solution (119) and by Zajta to develop a rapid method for investigating the stability of phenobarbital sodium solutions (120). Work carried out by Tishler, *et al.*, on phenobarbital degradation in solution was published (121). Nelson compared rate constants for sulfonamide acetylation *in vivo* with oil-in-water partition coefficient (122). Rates of oxidative destruction and alkaline saponification of some steroidal esters were determined—the sulfobenzoates, phosphates, and hexahydrobenzoates gave maximum stability (123). Schroeter stated that the reaction between bisulfite or sulfite ion and salicyl alcohol followed second-order kinetics over a wide pH range (124). Degradation of *l*-ascorbic acid solutions was found to be a pseudo-first-order reaction and dependent on pH and temperature (125). Kinetic studies were conducted by Rippie and Higuchi on the reaction between molecular oxygen and 2,3-dimercapto-1-propanol in aqueous solution (126).

Stability prediction, a very important part of pharmaceutical formulation, was reviewed in depth by Garrett (127). Scott and Lachman investigated the influence of nonequilibrium sample temperature on stability predictions extrapolated from elevated temperature studies (128). Stability prediction methods for liquid pharmaceuticals were reported (129). In a series of articles on pharmaceutical stability prediction, Garrett (130), Garrett and Royer (131), and Garrett and Umbreit (132) described detailed kinetic studies on steroids and antibiotics. The theory and practice of stability prediction in cosmetic formulation were treated by Lachman (133).

Degradation of a large number of therapeutically important compounds takes place by way of hydrolysis. Hence, knowledge of the kinetics of hydrolytic reactions is of importance in the formulation of stable pharmaceutical preparations. Windheuser and Higuchi (134) and Heathcote and Wills (135) have studied the kinetics of thiamine hydrolysis; the rate approximated a first-order reaction and was influenced by general base catalysis. In another paper, Finholt and Higuchi determined rates of hydrolysis of niacinamide (136). Kinetic studies on the hydrolysis of tropine esters were reported (137). Data published by Garrett and Weber showed that the stability of N-butylformamide to acid and enzymatic hydrolysis greatly exceeded any stomach retention time (138). Mitchell investigated the hydrolysis of ethyl benzoate, diethyl phthalate, and benzocaine in cetrimide solutions (139), and the hydrolysis of *p*-chlorobenzaldoxime has been related to its oral inefficacy (140). A kinetic study of the base-catalyzed hydrolysis of 3,5,5-trimethyl-2,4-oxazolidinedione was conducted (141). Suzuki employed chemical kinetics to evaluate the stability of aqueous succinylcholine chloride solutions (142, 143). Velocity constants were determined for hydrolysis of this choline derivative as a function of pH. A report on the hydrolytic kinetics of calcium acetylsalicylate carbamide in the presence of high concentrations of additives was published (144). Garrett has discussed the solvolysis of symmetrical and mixed aspirin anhydrides (145) and of 21-hydrocortisone esters and hemi-esters (146).

Vitamin Stability.—The general area of vitamin stability was reviewed by Parrak (147). Guillory and Higuchi investigated the solid state stability of some crystalline vi-

tamin A derivatives (148). Of the derivatives studied, vitamin A benzhydrazone and vitamin A succinate triphenylguanidine salt were the most stable. Other writers reported the effects of pH, antioxidants, metallic impurities, chelating agents (149), and thiamine hydrochloride (150) on the stability of liquid formulations containing vitamin A. The stability of cyanocobalamin in liver preparations used in the treatment of pernicious anemia was examined at different times and pH values (151). Spontaneous modifications of certain properties of vitamin B_{12}-Co^{60} during storage have been explored (152). Vitamin B_{12} stability in the presence of other vitamins has also been investigated (153, 154). Experimental work on the stability of some new thiamine derivatives was reported (155, 156), and various stabilizers for ascorbic acid in solution were compared (157).

Multivitamin preparations were the object of many stability investigations. Wai, *et al.*, studied the stability of vitamins A, B_1, and C in selected vehicles—formulation, method of combination, and manufacturing procedures were evaluated for their influence on stability (158). Also investigated were the stabilities of some polyvitamin products containing vitamins A_1, B_1, B_2, and C (159). The shelf life of different oral formulations containing vitamins B_1, B_2, B_6, and niacinamide was evaluated (160). In a preliminary report, Bonn, *et al.*, stated that some liquid multivitamin preparations were deficient in one or two ingredients before opening (161). Storage of vitamins B_1, B_2, B_6, and niacinamide was examined (162).

PHARMACEUTICAL TECHNOLOGY

This section of the review is primarily concerned with the technological aspects of pharmaceutical dosage forms. Cooper reviewed pharmaceutical technology in industrial pharmacy (163), while a similar review by Ullmann and Thoma dealt with pharmaceutical technology in research and education (164). Also reviewed were oral polio vaccine production (165), the technology of vitamin C (166), and micro-capsules—a new pharmaceutical dosage form (167). Progress in industrial mixing processes was reviewed by Baird (168). Barnett and James studied the particle-size distribution of marble after wet ball-milling and found that varying the solid-liquid ratio changed the mean particle size of the product (169). Some aspects of inhaler technology (170) and dermatological vehicle formulation (171) were reported. Faust has reviewed some of the newer materials used in cosmetics and dermatologic vehicles (172). Basic conditions for drying pharmaceutical residues have been investigated (173). Spray-drying of antibiotics and some other synthetic drugs (174) and the effectiveness of protective colloids in spray-drying of powdered flavoring materials (175) were evaluated. Selleri and Caldini studied some pharmaceutical compositions containing vitamin B_{12} and a new vitamin C derivative (176). They reported that gelatin capsules containing an anhydrous lipophilic substance provided better stability than syrups. The preparation and film-forming properties of synthetic glycerides were investigated by De Freitas (177).

Small amounts of belladonna tincture were made by a new extraction process for the preparation of tinctures (178). A study on the pH of pharmaceuticals dealt with various aspects of hydrogen-ion concentration and buffer capacity in tinctures (179). Kochhar and Lofgren studied the preparation of stable, sterile injections of physostigmine sulfate and salicylate (180). Sodium metabisulfite appeared to be the best stabilizer. Sciarrone explored some of the problems encountered in the preparation of isotonic solutions (181). Parenteral medication was the topic of two papers: one discussed parenteral solutions of vitamin B_{12} (182), while another described the use of wetting agents in the preparation of aqueous injectable solutions of oil-soluble vitamins (183). In another report, Gilroy and Mayne investigated the efficiency of a method for deaerating aqueous solutions (184).

Tablets.—The tablet is one of the most widely used forms of oral medication. Kovac reviewed its future with regard to automation and machinery (185, 186). Another discussion on automation suggested a punched-tape program for making tablets (187). Progress in coated and multilayered tablets was also reported (188). Research on tablet granulations was disclosed in the following three papers: in one, Perlman described the use of calcium sulfate granulations as a dry-blended tablet base (189); another proposed silicone oil as a good antiadherent in tablet granulations (190); and the third presented physicochemical studies on factors affecting granule strength and apparent granule density (191). Particle size of the powder was found to be an important element of both granule strength and apparent granule density. Some physical characteristics of compressed tablets were studied by Henderson (192).

Several articles on the disintegration of tablets were surveyed. One examined the influence of surfactants on the disintegration of tablets (193), and two others discussed the use of sodium bicarbonate–tartaric acid mixtures as disintegrating agents for tablets (194, 195). In still another, ten tablet disintegrants were evaluated in the formulation of tablets of two drugs of differing water solubility (196). Richter and Steiger-Trippi investigated tablet disintegration testing (197). The influence of the addition of mucilage to artificial gastric juice on tablet disintegration time has also been studied (198). In another study, the relationship between rate of dissolution and disintegration time was examined (199). Kaplan and Kish described a modification of the U.S.P. tablet disintegration apparatus (200). An apparatus was recommended by Krueger and Vliet for the *in vitro* testing of timed-release tablets and capsules (201).

A preliminary study of the temperature dependency of colorant loss in tablets was reported by Swartz, *et al.* (202), and Lachman, *et al.*, investigated the effect of ultraviolet absorbers on the photostability of colored tablets (203). Tablets of pyrilamine resin adsorbate with aspirin and vitamin C were more stable than control tablets containing the maleate salt of pyrilamine (204). Another paper discussed medicinal-Aerosil adsorbates in tablets (205). Castello and Mattocks studied the discoloration of tablets containing amines and lactose (206). No darkening occurred when amine salts were used with lactose. Of three emodin tablet formulas investigated, a lactose base provided the best stability (207).

Tablet coating with syrup (208) and the age stability of sugar coatings (209) were studied. Other researchers investigated the addition of moisture-resistant waterproof coatings to sugar-coated tablets (210, 211). In a series on protective coatings for tablets, Ida, *et al.*, studied amphoteric poly(vinylpyridine) derivatives (212). Another paper in this series reported research on the enteric coating properties of internally plasticized acrylic and methacrylic acid derivatives (213). Lappas and McKeehan described the use of synthetic polymers as enteric and sustained-release coatings (214). The use of cellulose acetate succinate (CAS) as an enteric coating for some compressed tablets was also reported (215). Using talc as a dusting powder, satisfactory tablets were obtained when CAS was applied from an acetone–ethyl acetate solution by a modified pan method. Fennell studied the development of an enteric coating (216), and enteric tablets prepared by coating or direct mixing have been described (217).

Emulsions.—In 1962 reviews were published on the theory (218) and on the structure and stability (219) of emulsions. Becher reviewed the theoretical aspects of emulsification with particular emphasis on factors affecting stability (220). Electrophoretic methods for evaluating various emulsions were studied (221), and dielectric constants of water-in-oil emulsions were employed in studying agglomeration (222). Higuchi, *et al.*, proposed a method for studying aggregation in oil-in-water emulsions (223). Other investigators explored the relationship between electrical resistance and dispersed-phase concentration of oil-in-water emulsions (224, 225). An equation was developed which would explain the results.

Many publications have appeared on the subject of emulsion stability. Riegelman discussed the theory of emulsions as applied to stability (226), and Riegelman and Pichon demonstrated that emulsion stability need not be related to the HLB values (227). A turbidimetric method for testing the stability of oil-in-water emulsions has been developed (228). In a quantitative theoretical study of the physical degradation of emulsions, Higuchi and Misra considered various factors governing the degradation rate of emulsions when the process is strictly molecular diffusion-controlled (229). An investigation of the dispersion and stability of several emulsions was published (230). Two investigations on the preparation of stable paraffin oil emulsions were also reported (231, 232).

Prediction of emulsion stability, including types of instability and test methods, has been discussed (233). The ultracentrifuge has been employed in studies relating to the stability of emulsions. In one study Garrett found the ultracentrifuge an excellent tool for evaluation and prediction of emulsion stability (234), and in another the ultracentrifuge was used for quantitative determination of emulsion stability (235). Still another, by Rehfeld, described a new rapid method for quantitatively studying the mechanical stability of emulsions using the ultracentrifuge (236). Coagulation of emulsions was separated into two distinct steps—namely, aggregation and coalescence.

Wachtel and La Mer have investigated the preparation and size distribution of some monodisperse emulsions (237). Phase inversion by homogenizer processing was explored by Shimamoto

(238). Factors affecting flavor loss after spray-drying of peppermint oil-acacia-water emulsions were also studied (239).

The use of various hydrocolloids in emulsions was reviewed by Schwarz (240). These materials have found their greatest use primarily as stabilizers. Ethoxylated higher fatty-alcohol emulsifiers for various types of emulsions have been investigated (241). Bogs and Naumann described an apparatus for measuring the strength of films around oil globules in emulsions (242). In another study by the same writers, viscosity determinations were employed in the evaluation of pharmaceutical emulsifying agents (243). Research on the development of fat emulsions for intravenous administration has also been published (244, 245).

Suppositories.—A penetrometer method for estimating the melting point of suppositories (246) and a method for measuring solution time of glycerin suppositories (247) were discussed. Another investigator explored deformation time and ability to liberate therapeutic substances as criteria for the evaluation of modern suppository bases (248). Setnikar and Fantelli developed an apparatus which reproduces conditions of the rectum for determining liquefaction time of rectal suppositories (249). The preparation and testing of "tropic proof" suppositories have been reported (250), and in a series on pharmaceutical preparations, Beral, *et al.*, have discussed the testing of suppositories (251). A rotational viscosimeter was employed in a study of the rheological properties of some suppository masses in the molten state (252). Measurements of *in vitro* activity were used to study the stability of chloramphenicol in several suppository bases (253).

Ointments.—The utility of methylcellulose (254), sodium carboxymethylcellulose (255), and water-soluble polyethylene glycol (256) as ointment bases has been reviewed. Mueller found that dextrans in combination with plasticizers produced good ointment bases (257). Belen, *et al.*, developed a modified vanishing cream base for chlorothymol ointments (258). The importance of the HLB system in ointments has been investigated (259). Also studied was the hydration of wool wax alcohols and their use as ointment bases (260). Determination of the water content of ointment bases (261) and rheological standardization of Hebra's lead ointment (262) have been reported. Steigleder and Raab discussed a new method for studying the protection by various ointments of the skin surface against contact with water (263). They found that ointments have prolonged influence on skin surface, even when protection from water is partially lost.

Suspensions.—Polderman has reviewed the use of suspensions as oral, topical, and injectable pharmaceutical vehicles—many references were cited (264). In another publication, Polderman discussed the physical chemistry of suspension systems with regard to stability, particle size, sedimentation, crystal growth, and zeta-potential (265). The grinding and evaluation of powders for suspension preparation were investigated (266), and a statistical investigation into the dispersibility of powdered drugs was conducted (267). Several advantages of using Cab-o-sil as a suspending agent were reported (268). Anderson and Plein published a pharmaceutical investigation on the geology, identification, and testing of selected Alberta bentonites (269, 270). Calamine lotion was the subject of two publications: one reviewed the application of bentonite as a suspending agent in calamine lotion (271); in the other, Swafford found that use of polyethylene oxide water-soluble resin as a suspending agent for calamine lotion produced a more elegant and more stable preparation (272).

Sterility.—Kirsch has reviewed new developments and problems in sterile pharmaceutical packaging (273). In a study of multiple-dose injectables, the effect of repeated withdrawals on sterility was investigated (274). The use of ethylene oxide gas in sterilization has been reviewed (275), and two more papers reported experimental work in chemical sterilization (276, 277). Gold studied sterilization of pharmaceutical preparations with ultrasonic energy (278). Steam sterilization of hollow containers has also been discussed (279). In another study, the sterilizing capacity of cationic exchangers in the preparation of demineralized water was investigated (280). Membrane filtration has been applied to testing the sterility of antibiotic materials (281).

Packaging.—Methods of testing pharmaceutical glassware have been reported (282, 283). Two papers discussed the utilization of plastics in pharmaceutical packaging (284, 285). In another, a comparison of different methods of washing, filling, and sterilization of ampuls was reported (286). Experimental data on rubber preparations and their effect on distilled water has been published (287). Strip packaging of pharmaceuticals was explored by Webb (288), and Frey considered packaging defects and methods of reducing them (289).

Aerosols.—A review with several references was published on the use of aerosols in the pharmaceutical field (290). An article by Sciarra and Eisen surveyed the formulation of various dermatological aerosols (291); while another, by Hart and Cook, reviewed emulsions as applied to various types of aerosols (292). Other papers discussed the advantages and problems of powder aerosols (293) and the improvement of aerosol packaging (294). The advantages of having a contract filler for aerosols were summarized by Peterson (295). Ruyssen reviewed the constitution and stability of foams (296). A report on the physical chemistry and stability of aerosols has also been published (297). As vehicles for bronchodilator drugs, Segal found aerosols to be of value for relief of bronchospasm (298).

PHYSICAL PHARMACY

Since its recognition in the late 1940's, physical pharmacy has grown to a place of prominence in pharmaceutical science. In this area, the principles of physics and physical chemistry are applied to the solution of pharmaceutical problems. Many papers were published on this theme in 1962.

Publications by Kamada on the applications of surfactants to pharmaceutical preparations described a temperature-scan method for studying solubilization (299, 300). Another investigator studied the interaction of pharmaceuticals with sucrose and hyprose esters (301). In a series of papers on the surface activation of medicinals, Utsumi and Harada (302–305), Harada (306), and Utsumi, *et al.* (307), explored the physical chemistry of several alkylsulfate derivatives. Salts of several basic drugs were prepared and examined for micelle formation, complexation, and solubility. Two film studies were presented: in one, some physical properties of interfacial films of potassium arabate were investigated (308); the other, by Kanig and Goodman, described evaluative procedures for pharmaceutical film-forming materials (309). Fuehrer (310) published a review on hydrogen bonds, and Huggins (311) discussed the physicochemical aspects of hydrogen bonds and their application to biology.

The validity and limitations of pH determinations (312) and the determination of activity coefficients with the glass electrode (313) were discussed. In another article, a nonlinear least-squares method was advocated for the calculation of activity coefficients from osmotic coefficient data (314). A discussion of the measurement and interpretation of dissociation constants of akaloids was published (315). Along this same line, Chatten studied the relationship between aqueous dissociation constants of organic bases and their half-neutralization potentials in organic solvents (316). A spectrophotometric method was employed for investigation of the acid dissociation constants of phenylalkanolamines (317). Chilton and Stenlake have investigated dissociation constants of some compounds related to lysergic acid (318), while Miyamoto and Brochmann-Hanssen investigated dissociation constants of certain γ-pyrone dicarboxylic acids (319).

A photoextinction-sedimentation method was recommended for the size analysis of barium sulfate powders (320). Gledhill has shown that particle-size distribution determination by turbidimetry is comparable with electron-microscopic analysis (321). Investigations of the particle-size distribution of monodispersed barium sulfate prepared by the EDTA method (322) and the physical properties of chloramphenicol particles (323) were reported.

As evidenced by the following reports, there is still considerable interest in the development of methods for studying medicament release from pharmaceuticals. Release of medication from various emulsified ointment bases was discussed by DeKay (324). An agar-plate test was used in studying the influence of different particle sizes of chloramphenicol on diffusion from a lipophilic hydrocarbon base (325). Wood, *et al.*, described a new diffusion cell for the measurement of salicylate diffusion within hydrophilic ointments (326). Ointment-base influence on the diffusion of other medicaments was also studied (327). In a study of various proflavine salts, Fenton and Warren found that the *n*-valerate exhibited the best diffusion from a cream base (328). Higuchi analyzed recently published data on medicament release from ointments and reported good agreement with theory (329). The diffusion of oxygen, nitrogen, and carbon dioxide through thin Teflon and silicone membranes has been investigated (330). In studying membrane permeability of various ions, Nakagaki, *et al.*, used a membrane electrode method (331). Membrane permeabilities, diffusion constants, and degree of association were determined for some phenoxazone compounds (332, 333).

Nogami and Nagai (334, 335) and Nogami, *et al.* (336), have studied the acid-neutralizing reaction of various antacid compounds, particularly dried aluminum hydroxide gel. They developed equations for acid-neutralizing velocity, one

for the calcium carbonate-type antacid and another for the dried aluminum hydroxide gel type. Experimental data demonstrated that the reaction between dried aluminum hydroxide gel and acid followed the proposed equation.

Solubility.—The solubility of solids in liquids is a very important aspect in the formulation of many types of pharmaceutical preparations. The literature of the physics and engineering of dissolution has been reviewed (337). Three monographs dealt with the relationship between agitation and dissolution. Larese, *et al.*, studied high-speed stirring techniques in solubility determination (338). At approximately 30,000 r.p.m. equilibrium is reached within 1 hour. Results were comparable with those obtained by conventional agitation. The second paper presented an investigation of the dissolution of particles in a stirred liquid (339). In the third, Hamlin, *et al.*, discussed loss of sensitivity in distinguishing real differences in dissolution rates due to increasing intensity of agitation (340). The relationship of chemical kinetics and dissolution was the subject of two investigations (341, 342). Reports of studies on the velocity of polymer dissolution were also found (343, 344). The relationship between *in vitro* dissolution rates, solubilities, and LT_{50}'s in mice for some salts of benzphetamine and etryptamine has been investigated—salt formation as a means of obtaining sustained release or prolonged action was also discussed (345).

An experimental method for studying growth rates of particles in aqueous media was developed by Higuchi and Lau (346). Electrical conductivity measurements were used to investigate the mode of action of solution adjuvants (347). Papers were published on the use of an approximate dielectric constant in solubility studies (348) and the correlation of solubility parameters and dielectric constants (349). The role of ionic strength, ion association, and solubility on the properties of electrolytes in solution has been reported (350). Another investigator studied the influence of inorganic salts on the solubility of organic pharmaceutical compounds (351). Levy and Procknal found that the dissolution rate of aluminum acetylsalicylate decreases with time because of the formation of a basic water-insoluble aluminum compound on the surface of the solid particles (352). Kato reported that glycols with hydroxyls on either terminal end or in 1,2 positions formed micelle-like structures in aqueous solution (353), and Mulley and Metcalf investigated the critical micelle concentration of some polyoxyethylene glycol monohexyl ethers in binary and ternary systems (354). In another solubility study, Higuchi and Misra examined the effect of solvent chain length on the solubilization of water by dioctyl sodium sulfosuccinate (355).

Investigations of the solubility of sucrose in aqueous mixtures of organic solvents (356) and the solubility of opium alkaloids in various organic solvents (357) were published. In another article, benzyl alcohol was suggested as a solvent for injectable solutions of quinine (358). Burnstine and Schmid found that the solubility of bilirubin in aqueous solutions was critically dependent on pH and ionic strength (359). Partition coefficients between water and butyl acetate were determined for various concentrations of benzylpenicillin (360). Debye and Coll used vapor-pressure measurements in studying the association of α-monoglycerides in nonaqueous solutions (361). Physicochemical investigations of aqueous sodium salicylate solutions were also reported (362, 363).

Two reviews on the solubilization of vitamins A and D were published (364, 365). Solubilization of benzoic acid derivatives in aqueous solutions with polyoxyethylene stearates was the subject of a monograph by Goodhart and Martin (366). Research on the solubilization of essential oils and oil constituents (367) and the use of amine salts of fatty acids for the solubilization of cholesterol (368) has also been published. Conductivity and surface tension measurements were used to study solubilizing properties of liquorice (369). Another report discussed the solubilizing ability of some polyethylene glycols and their esters (370).

Complexation.—The use of complexation in the stabilization of pharmaceuticals (371) and the application of chelating compounds in pharmacy and medicine (372) were reviewed. Walker has discussed the use of complexation in pharmaceutical systems (373). In another publication, Kennon and Chen presented a new approach to probability and complexation (374). They described and illustrated a method which considers the ability of the ionic atmosphere surrounding a drug in solution to form "probability complexes" with the drug.

Solubility experiments were used in studying the complexation of organic acids and bases with their salts in aqueous solution (375). A new molecular complex of tetracycline and urea was reported (376), and Guttman summarized results of a preliminary investigation of the effect of molecular interactions on velocities of reactions involving riboflavin (377). The stability of anionic complexes of some barbituric

acid derivatives and silver was studied by Leyda and Harris (378). Chelates of pyridine-2-aldoxime (379), metal chelates of mercapto-acid amides (380), and copper chelates of *l*-ephedrine (381) have been studied. Complex formation between boric acid and glycerin at elevated temperatures has been investigated (382). A method combining radioisotopes and ion exchange was used to study complexation of alkaline earth metals with citric acid and its derivatives (383). Metal complexes of nuclear-substituted salicylic acids and their biological effects were investigated by Foye and Turcotte (384). Two discussions on the preparation and properties of iron-carbohydrate complexes for injectable use were published (385, 386).

Surface Phenomena.—The importance of colloid and surface chemistry to biologically oriented and chemically oriented students has been reviewed—course content and methods of teaching were discussed (387–389). In a similar review, Wilson discussed the application of colloid and surface chemistry to problems in industrial research (390). Bailey, *et al.*, considered the meaning and function of average quantities in colloid science (391). They pointed out that the experimental method often determines the nature of the average obtained.

Barriers on the surfaces of dispersed particles (392) and the use of gelatin as a protective colloid (393) were the subjects of papers by two other investigators. In studies on the effect of deflocculating agents on suspensions of calamine and zinc oxide, Wood, *et al.*, measured particle size at various concentrations of sodium citrate (394). A new deflocculant and protective colloid for barium sulfate has also been reported (395).

Ikegami and Imai investigated the precipitation of polyelectrolytes by salts (396). Experiments on the flocculation of quartz and other suspensions with gelatin have been reported (397). Results indicated that gelatin must have a small positive charge for optimum flocculation of a negatively charged suspension. In his studies on the coagulation process, Packham investigated the effect of pH and turbidity (398). A new method was described which reduces pH variation. In a second paper Packham studied the effect of pH on the precipitation of aluminum hydroxide and measured the degree of precipitation by measuring the change in turbidity and the amount of residual soluble aluminum (399).

The sorption of water vapor by synthetic lecithin and cephalin has been studied (400). B-E-T plots showed that the synthetic compounds absorbed less than the corresponding natural compounds. An investigation of water vapor sorption and diffusion through hard gelatin capsules was also reported (401). A paper by Blank dealt with monolayer permeability and the properties of natural membranes; differences between bulk and monolayer processes were discussed (402). Methods for measuring gas adsorption on solids and their application to pharmaceutical products have been reviewed (403). Strickland studied water vapor sorption by pharmaceutical powders (404), and Gross examined the adsorption of some anticholinergic drugs by various antacids (405). Another investigator reported on the adsorption capacity of activated carbon (406).

Gillespie and Wiley considered the determination of London–van der Waals constants from suspension and emulsion viscosity and surface energy data (407), and other researchers employed solution adsorption in the measurement of specific surface areas of a wide variety of finely divided solids (408). In an experimental investigation of the behavior of a single droplet at an oil-water interface, Jeffreys and Hawksley found coalescence to be a stepwise phenomenon (409). Retention of liquids on solid surfaces was considered in two articles: a theory for spray retention based on the sliding of liquid drops on solid surfaces was developed in one (410); the other reported studies on the retention of aqueous suspensions on leaf surfaces (411). Surfactants were used for estimating the charges in gelatin at the isoelectric point (412). In a series on nonionic surface-active compounds, Becher studied the effect of electrolyte on micellar properties of some polyoxyethylene derivatives of lauryl and tridecyl alcohol and nonyl phenol in aqueous solutions (413). Another paper by Becher described a spectral dye method for the determination of critical micelle concentration (414). The effect of sucrose and cyclamate on the gel strength of gelatin, carrageenan, and algin has been studied (415). Another article reported the preparation of two-phase gels of iodinated poly(vinyl alcohol) (416).

Rheology.—In a series of papers on the subject of rheology in pharmacy, Lordi has discussed fundamental concepts (417), Newtonian flow (418), and non-Newtonian flow (419). Two papers on the swelling of polymer systems in solvents were published (420, 421): the first presented a method for obtaining complete swelling-time curves, while the second considered the mathematics of diffusion. Re-

view articles on the use of the Brookfield Synchro-Lectric viscometer for rheological investigation of pharmaceutical preparations (422) and theories of liquid viscosity (423) were also published. In the latter, Brush dealt with basic principles involved and some theories of current interest and cited almost 600 references. The role of viscosity and viscosity-enhancing materials in the compounding of pharmaceutical preparations has been discussed (424). Gold studied interfacial viscosity measurements and their relationship to selected emulsion systems (425).

A study of the coalescence of liquid drops in electric and shear fields was conducted (426). Umemura, in his studies on pharmaceutical dispersion, investigated the relationship between viscoelasticity and non-Newtonian flow (427, 428). In a third paper of this series, he extended the mathematical relationship of previous work to study temperature dependence of six dispersion systems (429). Haines has studied the caking of liquid dispersions (430). Theoretical calculations of sedimentation volume for the case of spherical particles attracted by cohesive force were presented (431), and Levine and Bell discussed an extension to the stability theory of lyophobic colloids (432). Effects of emulsification temperature and cooling rate on certain physical properties of a beeswax-mineral oil emulsion were studied by Boylan, *et al.* (433). Other researchers investigated the intrinsic viscosity of gelatin (434), and the influence of pH, concentration, time, dilute electrolyte, and functional groups on the gelation of gelatin and modified gelatins (435, 436).

Several papers were published on the rheological behavior of both aqueous (437) and organic (438–440) dispersions of Aerosil. Levy has investigated changes on aging of plain and polysorbate-80 containing dispersions (441). Another article by Levy dealt with the kinetics of structural recovery of thixotropic montmorillonite dispersions (442). In an investigation of unit layer interaction in hydrous montmorillonite systems, Van Olphen studied competition between double-layer repulsion forces and van der Waals attractive forces (443). The rheological behavior of clay-water systems was also discussed by Van Olphen (444). Application of the Verwey and Overbeek theory to the stability of kaolinite-water systems was described by Holtzman (445). A general method was presented for measuring adsorption of various anions on kaolinite.

Hydrodynamic studies on sodium carboxymethylcellulose in aqueous solutions have been conducted (446). An investigation was made of the rheological properties of corn oil emulsions with methylcellulose; a new equation was derived to explain flow properties (447). In another rheological study, consistency curves for procaine pencillin G aqueous suspensions were obtained at different concentrations (448). Non-Newtonian flow was observed in these suspensions at low shear rates, but Newtonian flow was found at high shear rates. The viscous behavior of a barium sulfate–water system has been studied (449). Rheological properties of aluminum stearate in benzene (450) and poly(ethylene oxide) in aqueous solutions (451) were investigated. Simonelli and Higuchi explored melting behavior, thermodynamic equilibrium, rate of crystal growth, and rate of melting of methyl stearate (452). Polymorphism in some pharmaceutically important lipid materials was examined by Eriksen, *et al.*, with the aid of heating and cooling curves (453).

PHARMACEUTICAL CHEMISTRY

For the purpose of this review, pharmaceutical chemistry is loosely defined as the area primarily concerned with the chemical aspects of pharmaceutical science. New drugs and drug products of 1961 (454, 455), principles and chemistry of central analgesics (456), crystallography (457, 458), and the use of quaternary ammonium compounds in medicinal chemistry (459, 460) were subjects of review articles published in 1962. A table of melting points of medicinals and index of synonyms has also been published (461). Melting points from 30 to 351° were listed. Several additional publications discussed properties and uses of enzymes in pharmacy and medicine (462–466). In another study on enzymes, the effect of sulfate content of anionic polymers on the *in vitro* activity of pepsin was investigated (467).

Surfactants were the subject of considerable research. Raphael reviewed detergents and surface-active agents (468). The surface activity of glycerol ethers (469) and some synthetic nonionic detergents (470) has been studied. Applications of polysorbate 60 in pharmaceutical compounding were described (471). Ullmann and Moser investigated the effect of polyoxyethylene-adducts on the antibacterial activity of antibiotics (472).

Many investigations have been published on phenothiazine derivatives. Borg and Cotzias examined structure-reactivity correlations, formation of free radicals, and the theory of the interaction of trace metals with phenothiazine deriva-

tives (473–475); and photodecomposition of thioproperazine was investigated by Yamamoto and Fujisawa (476). Other investigators have studied reduction products of santonin (477) and some difficultly hydrolyzable flavonosides (478). Ugai prepared codeine phosphate crystals with 0.5, 1, and 1.5 molecules of water of crystallization in stable form (479). Structural modifications in the search for new drugs (480) and the chemistry of thiamine and allied compounds (481) have been reviewed. Campbell (482) and Campbell and Slater (483) reported the alteration of physical properties of some therapeutic agents through the formation of N-cyclohexylsulfamate-drug salts. Several such drug-salts were prepared and evaluated; both taste and stability were good. Correlation of taste and structure of α-D-mannose and β-D-mannose was discussed (484). The chemistry and properties of a new plasma expander were also described (485).

Antibiotics.—Almost 400 references were cited in a review on the chemistry and pharmacology of antibiotics from 1956 to 1961 (486). The chemistry, pharmacy, and pharmacology of pencillin were also reviewed (487, 488). Synthesis of C^{14}-benzylpenicillin for tracer studies from C^{14}-phenylacetic acid and 6-aminopenicillanic acid was described by Nettleton, *et al.* (489). Several tasteless chloramphenicol derivatives have been prepared and evaluated—the best compound appeared to be the dodecylsuccinate (490). Research on properties and chemical purification of nystatin was reported (491).

The tetracycline antibiotics have been the subject of considerable experimental effort. Stempel has reviewed their development (492). Three different papers were concerned with tetracycline production and purification methods (493–495). The isolation and characterization of two new tetracycline antibiotics (496) and a new soluble tetracycline (497) have been reported. Remmers, *et al.*, described a new alkaline-stable species for selected members of the tetracycline family (498). Extensive use of half-life was made in measuring stability of the new derivatives. Demethylchlortetracycline was reviewed by Gill (499).

Ion Exchange.—The applications of ion-exchange resins in cosmetics, medicine, and pharmacy have been reviewed (500, 501). Hirscher and Miller discussed drug release from cation-exchange resins (502), and Schlichting described preparation, assay, and release rates of six new carbinoxamine cation-exchange resin salts (503). An experimental test of the theory of particle-diffusion controlled ion-exchange was reported (504). Bonner, *et al.*, employed measurements of ion-exchange equilibria as a rapid and convenient method for estimation of ionization constants of acids in the 10^{-3} to 10^{-1} range (505). The behavior of ion-exchange resins in aqueous ammonia solutions (506) and the behavior of ascorbic acid on redox ion-exchange resins (507) were studied. Adsorption of vitamin B_{12} by various cation exchangers was discussed by Morita and Tanaka (508). Carboxylic cation-exchange resins were used for the separation and purification of antibiotics (509). Ion-exchange resins have also been employed for the extraction of codeine (510) and the removal of inorganic salts from concentrated streptomycin solutions (511). In another study, ion-exchange resins and electrodialysis were combined for isolation and purification of mixtures of plant alkaloids (512).

Polymers.—In a study of the constitution of alginic acid, Drummond, *et al.*, confirmed the presence of 1,4′-linked L-guluronic acid units (513). Schweiger prepared some algin acetates and studied their viscosity (514) and reaction with calcium and other divalent ions (515). He concluded that gelation or precipitation of alginates with calcium ions occurs through a complex involving two carboxyl groups from neighboring units and two hydroxyl groups in a unit of probably another chain. Another investigator described conditions for the isolation of alginic acid from algae of the Adriatic Sea (516). Equilibrium dialysis studies were used in determining the extent of binding of alkaloids to carrageenan and other hydrocolloids (517).

Studies on the electrochemistry of azrechtic acid were published (518). Murty, *et al.*, studied the physical chemistry of water chestnut starch (519), while Greenwood and Thomson described the fractionation and characterization of starches from various plant origins (520). Complexes of pepsin with sulfates of oxidized starch and its reduced products have been reported (521). Cohen examined the interaction of pharmaceuticals with the Schardinger dextrins (522), and Blatz described the synthesis and preliminary characterization of new polyelectrolytes (523). Thermally stable salts of poly(vinylphthalic acid) were also investigated (524). The interactions between certain chemicals and some water-soluble macromolecules were studied (525, 526).

Methodology.—Paper chromatographic procedures were published for the characterization of antibiotics (527) and the separation of

codeine, morphine, and nalorphine (528). Brochmann-Hanssen and Svendsen described an extremely sensitive method for the separation and identification of barbiturates and some related compounds by means of gas-liquid chromatography (529). Thin-layer chromatography has been used to study the identity and purity of some fats and oils (530) and for separation of some pharmaceutically important corticosteroids (531). The preparation of microcrystalline progesterone using ultrasound was described by Principe and Skauen (532). Methods for the separation of sterols by countercurrent crystallization (533) and the purification of organic compounds by continuous zone freezing (534) have been reported. Automatic pH control of the preparation of medicinal compounds has been investigated (535).

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[1] *Index Medicus* abbreviations were used for journals not listed in *Chemical Abstracts*. In a few instances where the writer was unable to obtain late 1962 issues, references were obtained from *Current Contents*.

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Dosage Variation in Tablets

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Tablet granulations of phenobarbital and reserpine were prepared on a routine manufacturing basis and compressed on a single-punch tablet press. The tablets were collected in small groups in the order of compression, weighed, and analyzed individually. Statistical quality control charts were prepared for weight variation and per cent composition, the main variables affecting the dosage variation. Inspection of the control charts and results obtained by application of the *F*-test indicated that some disruption of uniformity occurred during the compression of the tablets.

THE TOLERANCE LIMITS in the "United States Pharmacopeia" and "National Formulary" are established in such a way as to make allowance for a number of unavoidable variables, including the assay error, the sampling error, and a certain amount of deterioration of the active ingredient (1, 2). In an effort to reduce the sampling error for tablets, the official compendia specify that not less than a stated number of tablets be taken as the sample. Although this number frequently may be as low as 10 or 20, it may run as high as 200 for tablets of certain potent drugs. The result of the assay expresses the average content of active ingredient per tablet in the sample but tells nothing about the uniformity of dosage. There are two obvious sources of variability affecting the drug dosage in tablets—namely, the variation in weight and the variation in per cent composition. The variation in weight has been extensively studied by many investigators (3–5) and can be determined and controlled by the official method or, better, by a method based on the standard deviation. The control chart is a useful tool for controlling the weight variation during production. Composition variation is not as easily determined. Studies carried out in several laboratories have indicated that this variation often is the largest of the two.

Variation in per cent composition is associated with the problems of mixing. Although there is now an appreciable literature on the mixing of solids in general, it is only in recent years that the pharmaceutical aspects of mixing have been studied and their effects on the uniformity of drug dosage and drug standardization have been recognized. In the production of tablets, a perfect mixture represented by a random distribution of the particles can never be attained. Proper equipment and sufficient time of mixing are certainly important considerations, but the mixing process is also affected by the size, shape, proportion, and density of the particles and by various surface-active forces (6, 7). Even if a fairly uniform particle distribution has been achieved, this condition is unstable and is easily disturbed by vibration or bumping (7). In the preparation of uniform tablet granulations, the solubility of the drug in the granulating solvent, and the conditions of drying also play important roles (8).

The various problems associated with the mixing of solids together with the minimum sample required for analysis of tablets have led Train (7) to predict that, although the official specifications may be met under the conditions of the assay, the dosage variation can be over twice the official limits in a few cases, over four times the official assay limits in most cases, and eight or more times the official limits with one or two formulations. This is especially true if the active ingredient is less than half the tablet weight and is added as a powder or a batch of concentrated granules which are mixed with other granular materials.

The effect of the particle size and the proportion of active ingredient on the uniformity of powder mixtures, granulations, and compressed tablets have been studied by Fryklöf (9) and Banker, Christian, and DeKay (8). Commercial samples of various tablet preparations analyzed by the Food and Drug Administration showed a wide range of dosage apparently not related to the weight variation (10). Moskalyk, *et al.* (11), reporting on the uniformity of drug dosage in commercial tablets, have also found

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variations in potency which have been greater than those indicated by the weight variation.

Raff, *et al.* (12), have shown that internal flow and segregation of granulation take place during compression, causing a change in the weight and the hardness of the tablets. It is of interest to know to what extent such segregation affects the composition of the tablets. The present investigation was undertaken with this in mind and also for the purpose of studying the relationships between dosage variation and composition variation for tablets containing various concentrations of active ingredient.

EXPERIMENTAL

Tablet Preparations.—Phenobarbital tablets were prepared in three different strengths—16 mg., 65 mg., and 100 mg. per tablet—the drug constituting approx. 10, 33, and 44% of the tablet weight, respectively. A batch of 0.25 mg. reserpine tablets was also included in which reserpine amounted to about 0.175% of the tablet weight. The tablet granulations were prepared according to standard manufacturing procedures by wet granulation of mixtures of the drug and the diluents with aqueous granulating agents. The tablet press was a Colton model 2B single-punch machine operated at a speed of 90–100 tablets per minute. It had been in operation for many years and was worn to the extent of having a somewhat rough action. The batches which ranged in size from 60,000–300,000 tablets were produced on a routine basis in the Pharmaceutical Technology Laboratory for the University Hospital Pharmacy.

Sample Collection.—The hopper was filled, the machine adjusted for correct weight and pressure, and allowed to run for about 0.5 hour. From then on, samples of five tablets each were collected at 30-minute intervals; the tablets were weighed and assayed individually. This was done independently of the checking for weight variation and the normal adjustments made by the manufacturing personnel.

To study the uniformity of the tablet granulations, small samples were collected from the bulk granulation during the course of the tableting process. Amounts approximately equal to the tablet weight were weighed out and analyzed as described below.

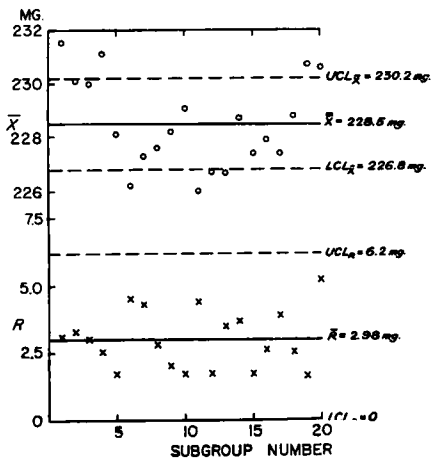

Fig. 1.—Control chart for weight variation of 100-mg. phenobarbital tablets.

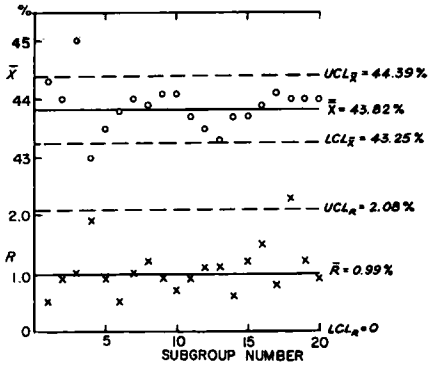

Fig. 2.—Control chart for the variation in per cent composition of 100-mg. phenobarbital tablets.

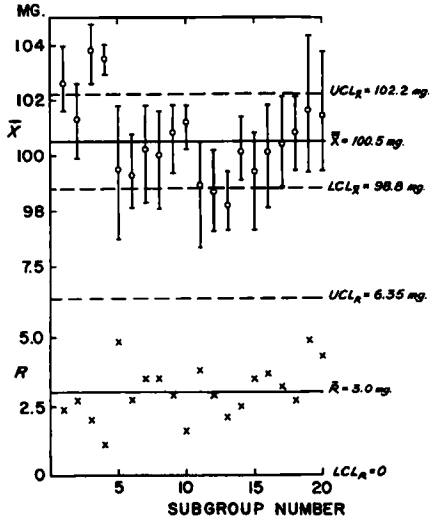

Fig. 3.—Control chart for dosage variation of 100-mg. phenobarbital tablets.

Assay Procedures.—Because of the large number of determinations to be made and the low dosage of some of the tablets, it was essential to have assay procedures which were rapid and sensitive and had a high degree of precision. Individual phenobarbital tablets were placed in 100-ml. volumetric flasks containing 25 ml. of water. After disintegration of the tablets, ethanol (95%) was added to volume. When the drug had dissolved, the solution was thoroughly mixed and filtered through a dry filter; the first 25 ml. of the filtrate was discarded. An aliquot portion of the filtrate was mixed with borate buffer of pH 10, diluted with water to an appropriate concentration, and the absorbance was read in a Beckman model DU spectrophotometer against a reagent blank at 240 mμ. Phenobarbital from the same production batch was used as the reference standard.

Reserpine tablets were also assayed by ultraviolet spectrophotometry. One tablet was placed in a 25-ml. volumetric flask, 2 ml. of water was added, and the tablet was allowed to disintegrate. After

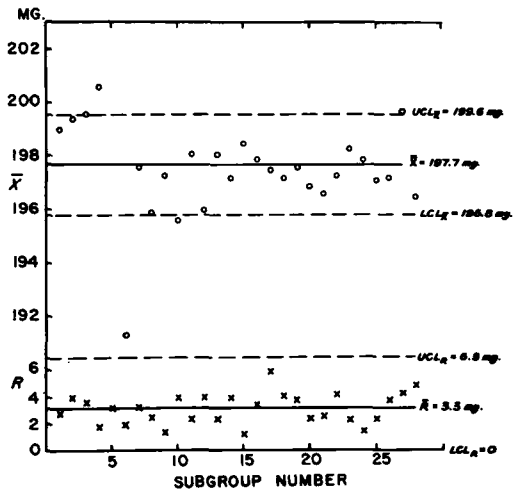

Fig. 4.—Control chart for weight variation of 65-mg. phenobarbital tablets.

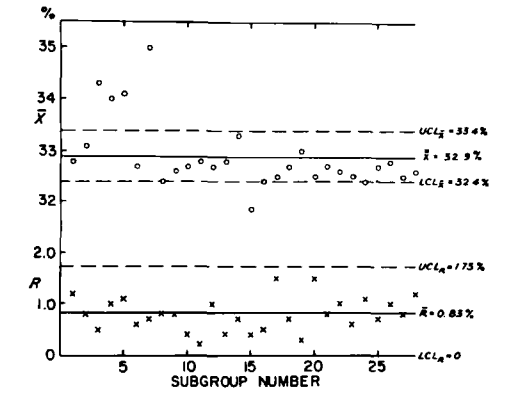

Fig. 5.—Control chart for the per cent composition variation of 65-mg. phenobarbital tablets.

addition of 20 ml. of reagent grade methanol, the mixture was heated gently on a steam bath for 5 minutes and then cooled to room temperature. Methanol was added to volume, the solution mixed well and filtered through a small dry filter; the first 10 ml. of the filtrate was discarded. The absorbance of the filtrate was determined at 268 mμ against a mixture of 2 ml. water and 23 ml. methanol.

Precision.—For the determination of precision, accurately weighed amounts of the reference standards were dissolved and treated in the same manner as the tablets. The standard deviation was calculated on the basis of 15 independent determinations. The coefficient of variation was 0.80% for the phenobarbital assay and 2.5% for the reserpine assay.

Control Charts.—The control chart designed by Shewhart, a commonly used tool for process control, is based on the two types of variables involved in the manufacturing process. One is the random variability associated with short-term variability in the raw material, handling, measurement, etc. The other is the variability due to real changes in the process level. The random variability is estimated on the basis of the variability within small subgroups collected in short periods of operation during which only random errors, but no real changes in process level, are likely to occur. Three-sigma limits are usually drawn about an established standard level. If there is no shift in the process level, 99.73% of the subgroup averages would be expected to fall within these control limits. The commonly used control charts for variables are the $\bar{X}$ and the R charts, showing shifts in the process average and the dispersion, respectively. The statistical methods for preparation and use of the control charts are given in standard texts (13, 14).

RESULTS AND DISCUSSION

Figure 1 illustrates the $\bar{X}$ and R charts for weight variation of 100-mg. phenobarbital tablets. The range shows evidence of control during the whole period of production, whereas the process average does not. This is partly due to the rough action of the tablet machine and segregation of the particles in the granulation. The variation in per cent composition for the same tablets is illustrated in Fig. 2. It is apparent that the system is not in control from a statistical point of view. Instead of being randomly distributed around the batch mean, the process average shows a cycling effect. The two sources of variation are combined in Fig. 3 which gives the actual dosage variation in terms of content of phenobarbital per tablet. The vertical lines in the $\bar{X}$ chart indicate the spread of individual tablets about the subgroup means. None of the 100 tablets analyzed during the compression of this batch deviated more than ±4% from the batch average or more than ±4.5% from the labeled amount.

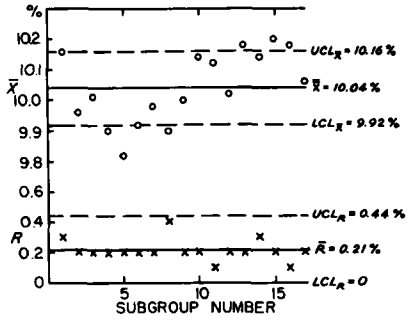

Fig. 6.—Control chart for the per cent composition variation of 16-mg. phenobarbital tablets.

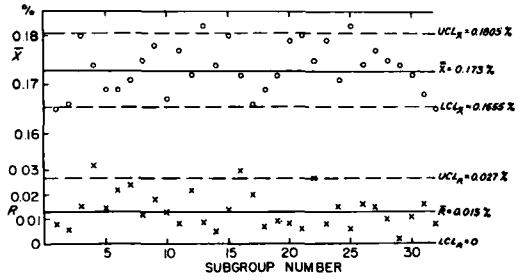

Fig. 7.—Control chart for the per cent composition variation of 0.25-mg. reserpine tablets.

TABLE I.—COEFFICIENTS OF VARIATION FOR TABLET WEIGHT, POTENCY, AND PER CENT COMPOSITION OF FOUR BATCHES OF TABLETS

Tablet Batch	Coefficient of Variation—Tablet Wt.	Dosage	Composition, %
Phenobarbital, 100 mg.	0.87	1.87	1.38 (1.12[a])
Phenobarbital, 65 mg.	1.21	2.84	2.32 (2.17[a])
Phenobarbital, 16 mg.	1.14	2.02	1.44 (1.20[a])
Reserpine, 0.25 mg.	2.01	4.86	4.43 (3.65[a])

[a] This value does not include the assay error.

The control charts for 65-mg. phenobarbital tablets are shown in Figs. 4 and 5.

After subgroup 12 had been collected, the hopper was refilled regularly every 30 minutes when it was about half full. This resulted in much better control of the weight variation (Fig. 4). The tablet composition showed considerable lack of uniformity during the early part of the operation. It improved later, but there was an apparent cycling tendency during the production schedule in the same way as shown in Fig. 2. Of the 140 tablets analyzed from this batch, only ten exceeded the limits of 100 ± 6% of the labeled amount, and all ten tablets came from the first 12 subgroups. Beginning with subgroup 13 no tablet deviated from the labeled amount by more than ±5%.

The per cent composition variation of 16-mg. phenobarbital tablets is illustrated in Fig. 6. Although the composition is not in control in a statistical sense, the variability is still relatively minor. A total of 85 tablets were analyzed from this batch, and no tablet deviated from the labeled amount by more than ±5%.

Figure 7 gives the control charts for the composition variation of 0.25-mg. reserpine tablets. A total of 160 tablets were analyzed individually and only six tablets fell slightly outside the limits of 100 ±10% of the batch average.

A comparison of the coefficients of variation for weight, potency, and per cent composition of the four tablet batches is given in Table I. The apparent variabilities calculated on the basis of the experimental results include the errors of measurement along with the actual variability in the tablets. Although the weighing error is very small and may be ignored in this connection, the assay error is appreciable, particularly for reserpine. In Table I the coefficients of variation in parentheses express the actual variability of the per cent composition (V'), calculated from

$$V'_{\text{compn.}} = \sqrt{V^2_{\text{compn.}} - V^2_{\text{assay}}}$$

The variation in composition was of approximately the same magnitude as the variation in weight in two of the tablet batches. The composition variation was the greatest in the other two batches. As would be expected, there was a tendency for the mixing error to increase as the percentage of the drug in the granulation decreased. This tendency is not always apparent when commercial tablets are studied because greater care is usually exercised in the mixing process when the drug is in low concentration. Generally speaking, the greatest variability in the tablet composition occurred early in the production schedule, after which the process appeared to settle down to a more or less regular cycle. This is not only due to a lack of homogeneity on the part of the granulation, but probably also due to a change in its composition during the compression of the tablets. Samples of granulation collected from the bulk container during the compression schedule gave a variance which by the F-test was significantly smaller than the variance of the tablets ($P = 0.05$). The vibration taking place in the hopper of the tablet press might cause many of the original granules to break up into smaller particles; they would pass through the spaces between the larger particles together with the finely powdered lubricant and disintegrating agents, thus changing the composition.

Banker, Christian, and DeKay (8) have shown that uniformity of a tablet granulation can be improved if a granulating solvent is used in which the drug is soluble. The aqueous granulating agents used for the tablets described in our work are poor solvents for phenobarbital and reserpine. In view of this handicap, the uniformity achieved in the tablet composition appears to be quite satisfactory.

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Synthesis of DL-α-(Dihexanoyl-, Dioctanoyl-, and Didecanoyl)-Phosphatidyl DL-Serines

By JONAS MAURUKAS and STEPAS KAIRYS

Condensation of monophenylphosphoryl N-carbobenzyloxy DL-serine benzyl ester monosilver salt with the appropriate α-iodo diglyceride by the procedure of Bevan, Malkin, and Tiplady for the synthesis of racemic α-(distearoyl)-phosphatidyl serine, afforded dihexanoyl-, dioctanoyl-, and didecanoyl-DL-α-glycerylphenylphosphoryl N-carbobenzyloxy DL-serine benzyl esters. These esters, upon removal of the protective phenyl, benzyl, and carbobenzyloxy groups by catalytic hydrogenolysis, gave the short-chain fatty acid, DL-α-phosphatidyl DL-serines. In contrast to α-(distearoyl) phosphatidyl serine, these three phosphatidyl serines easily form an emulsion with water and should prove to be suitable substrates for biological and chemical studies.

SYNTHETIC PROCEDURES are available for the preparation of phosphatidyl serine containing a long-chain fatty acid (1, 2). However, investigation of the biological properties and effects of this compound is hampered by its insolubility in water and by the difficulty with which it forms aqueous emulsions (3). The work presented here describes the synthesis of phosphatidyl serines containing short-chain fatty acids. Such serines can be easily emulsified; therefore, they may be of value in biological investigations.

The synthesis reported in this paper utilizes the monophenylphosphoryl N-carbobenzyloxy DL-serine benzyl ester monosilver salt described by Bevan, Malkin, and Tiplady (2). The use of the monosilver salt eliminates the possibility of the formation of bis-phosphatidic acid which is obtained as a by-product in the method of Baer and Maurukas (1). The procedure shown in the reaction scheme is as follows: N-carbobenzyloxy DL-serine benzyl ester (I) was phosphorylated with phenylphosphoryl dichloride in the presence of anhydrous quinoline and anhydrous ethanol-free chloroform in a −10° bath. The product, consisting of phenylphosphoryl N-carbobenzyloxy DL-serine benzyl ester chloride (II) and bis-(N-carbobenzyloxy DL-serine benzyl ester)-phenyl phosphate (III), was caused to react further with potassium carbonate in water and pyridine to yield phenylphosphoryl N-carbobenzyloxy DL-serine benzyl ester monopotassium salt (IV). The potassium salt was converted into the monosilver salt V and purified by crystallization from boiling 95% ethanol. Condensation of DL-α-iodo diacylglycerol (VI) with phenylphosphoryl N-carbobenzyloxy DL-serine benzyl ester monosilver salt (V) in boiling xylene yielded diacyl-DL-α-glycerylphenylphosphoryl N-carbobenzyloxy DL-serine benzyl ester (VII). Hydrogenolysis in the presence of mixed platinum and palladium catalyst yielded phosphatidyl serines VIII in overall yields of 57–62%. This procedure should also prove suitable for the synthesis of both enantiomeric forms of the phosphatidyl serines.

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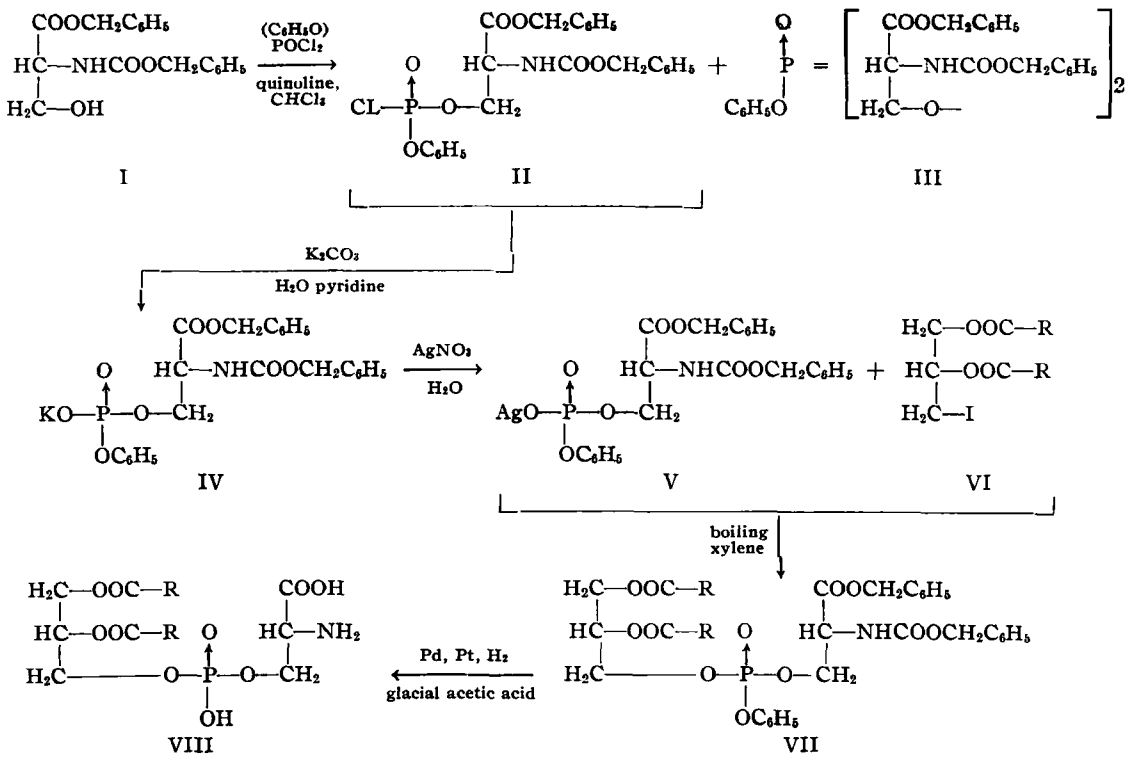
EXPERIMENTAL

DL-α-(Dihexanoyl)-phosphatidyl DL-Serine

Condensation.—Five Gm. (8.46 mmoles) of phenylphosphoryl N-carbobenzyloxy DL-serine benzyl ester monosilver salt (2), 85 ml. of boiling anhydrous xylene, and 2.70 Gm. (6.78 mmoles) of DL-α-iodo dihexanoylglycerol[1] were placed in a 200-ml. two-necked round-bottom flask equipped with a reflux condenser and mechanical stirrer. The mixture was stirred and refluxed in the dark for 15 minutes with the exclusion of moisture. After a short cooling period under running tap water, the mixture was centrifuged to remove silver iodide, and the clear yellowish supernatant was evaporated under reduced pressure at a water bath temperature of 35–40°. The resulting oil (6.5 Gm.) was dissolved in 150 ml. of ether and washed successively with saturated sodium bicarbonate solution and water until the water was neutral to litmus. After drying over anhydrous sodium sulfate, the ethereal solution was evaporated to dryness under reduced pressure at a bath temperature not exceeding 40°. The residue was suspended in 40 ml. of warm petroleum ether, refrigerated for 1 hour at 0°, and separated from the supernatant petroleum ether by centrifugation. The residue was dried *in vacuo* (0.025 mm.) to constant weight. The dihexanoyl-DL-α-glycerylphenylphosphoryl N-carbobenzyloxy DL-serine benzyl ester, a viscous oil, weighed 4.0 Gm. (78.0% of theory). At room temperature this compound was highly soluble in ether, acetone, methanol, ethanol, ethyl acetate, and acetic acid, but insoluble in petroleum ether and water. For analysis, 0.5 Gm. of this product was further purified by dissolving in 5 ml. of chloroform

[1] The three required α-iodo diglycerides containing hexanoic, octanoic, and decanoic acids have not been described in the literature. They were prepared from racemic α-iodo glycerol (4) *via* acetone glycerol (5) and esterified with the appropriate fatty acid chloride as described for the preparation of α-benzyl ether didecanoyl glycerol (6).

Reaction Scheme



DL-α-(diacyl)-phosphatidyl DL-serine
R = C_5H_{11}, R = C_7H_{15}, R = C_9H_{19}

and passing it through a chromatographic column (20 × 380 mm.) containing a mixture of 20 Gm. of silicic acid (Merck) and 10 Gm. of Celite (Johns-Manville).[2] The substance was eluted with 200 ml. of chloroform; the solvent evaporated to dryness at reduced pressure and at a water bath temperature not exceeding 40°. The recovery of dihexanoyl-DL-α-glycerylphenylphosphoryl N-carbobenzyloxy DL-serine benzyl ester was 0.46 Gm., N_D^{25} 1.5158.

Anal.—Calcd. for $C_{39}H_{50}NO_{12}P$ (755.78): C, 61.97; H, 6.67; N, 1.85; P, 4.10. Found: C, 61.20; H, 6.50; N, 1.76; P, 4.08.

Catalytic Hydrogenolysis.—A solution of 4.0 Gm. of dihexanoyl-DL-α-glycerylphenylphosphoryl N-carbobenzyloxy DL-serine benzyl ester in 75 ml. of glacial acetic acid with 3 Gm. of platinum oxide-palladium black catalyst (1 : 1) (7, 8) was placed in an all-glass hydrogenation vessel of 200-ml. capacity. The mixture was shaken vigorously in an atmosphere of pure hydrogen at an initial pressure of 40 cm. of water until the absorption of hydrogen ceased. One liter was consumed in approximately 2 hours. Hydrogen was replaced by nitrogen, an additional gram of mixed catalyst was added, and hydrogenation was continued until absorption ceased. Approximately 0.8 L. of hydrogen was consumed in 1 hour. Hydrogen was again replaced by nitrogen. The catalyst was removed by centrifugation and washed with a small amount of glacial acetic acid. The supernatant solutions were combined, and the acetic acid was removed by distillation under reduced pressure at a bath temperature of 35–40°. To complete the removal of traces of acetic acid, the substance was dissolved in 25 ml. of ether, the solution was diluted with 100 ml. of petroleum ether, and placed in a refrigerator at 0°. After 1 hour the crystalline material was centrifuged, washed with a small volume of ice-cold petroleum ether, and dried to constant weight *in vacuo* (0.025 mm.) over sodium hydroxide. The DL-α-(dihexanoyl)-phosphatidyl DL-serine weighed 1.93 Gm. (80% of theory); overall yield based on α-iodo diglyceride, 62.5%; m.p. 100°.[3] This waxy substance at room temperature is highly soluble in ether, chloroform, acetone, methanol, ethanol, ethyl acetate, and acetic acid, but insoluble in petroleum ether. It readily forms an emulsion in water.

Anal.—Calcd. for $C_{18}H_{34}NO_{10}P$ (455.44): C, 47.46; H, 7.53; N, 3.07; P, 6.80. $C_{18}H_{34}NO_{10}P$. Found: C, 47.29; H, 7.47; N, 3.02; P, 6.77.

DL-α-(Dioctanoyl)-phosphatidyl DL-Serine

DL-α-(dioctanoyl)-phosphatidyl DL-serine was prepared as described for the C_6 *homolog*. The condensation of 10.2 Gm. (17.3 mmoles) of phenylphosphoryl N-carbobenzyloxy DL-serine benzyl ester

[2] The mixture of silicic acid and Celite was washed successively with methanol and chloroform before placing it on the column.

[3] Melting points reported in this work were determined on the Fisher-Johns melting point apparatus.

monosilver salt with 6.2 Gm. (13.2 mmoles) of DL-α-iodo dioctanoylglycerol in 170 ml. of boiling dry xylene yielded 9.1 Gm. (82% of theory) of dioctanoyl-DL-α-glycerylphenylphosphoryl N-carbobenzyloxy DL-serine benzyl ester. This viscous oil at room temperature is highly soluble in acetone, methanol, ethanol, ethyl acetate, chloroform, ether, petroleum ether, and acetic acid, and is insoluble in water.

For analysis, 0.5 Gm. of substance was placed on a chromatographic column described for the C_6 *homolog* and was eluted with 210 ml. of chloroform. The recovery of analytically pure dioctanoyl-DL-α-glycerylphenylphosphoryl N-carbobenzyloxy DL-serine benzyl ester was 0.47 Gm.; N_D^{25} 1.5058.

Anal.—Calcd. for $C_{43}H_{58}NO_{12}P$ (811.88): C, 63.61; H, 7.20; N, 1.73; P, 3.82. Found: C, 63.51; H, 7.41; N, 1.71; P, 3.76.

The hydrogenolysis of 9.1 Gm. of dioctanoyl DL-α-glycerylphenylphosphoryl N-carbobenzyloxy benzyl DL-serine ester yielded 6.07 Gm. of crude phosphatidyl serine. This oil (containing traces of acetic acid) was dissolved in 116 ml. of warm ether and was precipitated with 350 ml. of petroleum ether. After 16 hours of refrigeration at −12°, the precipitate was centrifuged, washed with ice-cold petroleum ether, and dried *in vacuo* (0.025 mm.) over sodium hydroxide. The dioctanoyl-DL-glycerylphosphoryl DL-serine (weighing 4.0 Gm.) was obtained in a yield of 69.5% of theory (overall yield 57.0%), m.p. 160–161°. At room temperature this phosphatidyl serine was very soluble in chloroform, acetone, methanol, ethanol, ethyl acetate, acetic acid, and benzene, but insoluble in ether and petroleum ether. It readily formed an emulsion in water.

Anal.—Calcd. for $C_{22}H_{42}NO_{10}P$ (511.55); C, 51.85; H, 8.28; N, 2.74; P, 6.06. Found: C, 51.68; H, 8.36; N, 2.73; P, 6.02.

DL-α-(Didecanoyl)-phosphatidyl DL-Serine

DL-α-(didecanoyl)-phosphatidyl DL-serine was prepared as described for the C_6 *homolog*. The condensation of 10.0 Gm. (16.9 mmoles) of phenylphosphoryl N-carbobenzyloxy DL-serine benzyl ester monosilver salt with 7.0 Gm. (13.6 mmoles) of α-iodo didecanoylglycerol in 170 ml. of boiling dry xylene gave 9.5 Gm. (80% of theory) of didecanoyl-DL-α-glycerylphenylphosphoryl N-carbobenzyloxy DL-serine benzyl ester. At room temperature it was readily soluble in acetone, methanol, ethanol, ethyl acetate, chloroform, ether, petroleum ether, and acetic acid, but insoluble in water. For analysis, 0.5 Gm. was passed through a silicic acid chromatographic column as described for the C_6 *homolog*. The recovered substance weighed 0.44 Gm.; N_D^{25} 1.5030.

Anal.—Calcd. for $C_{47}H_{66}O_{12}NP$ (867.99): C, 65.03; H, 7.67; P, 3.57; N, 1.61. Found: C, 64.51; H, 7.57; P, 3.52; N, 1.56.

The catalytic hydrogenolysis of 9.5 Gm. of didecanoyl-DL-α-glycerylphenylphosphoryl N-carbobenzyloxy-DL-serine benzyl ester yielded 5.0 Gm. of a glass-like phosphatidyl serine. This product (containing trace amounts of acetic acid) was dissolved in 115 ml. of petroleum ether and was evaporated to dryness. The amorphous residue was recrystallized from 47 ml. of absolute ethanol by immersion in an acetone dry ice bath at −40°, centrifuged, washed several times with cold ethanol, and dried *in vacuo* over sodium hydroxide. The final product, DL-α-(didecanoyl)-phosphatidyl DL-serine, weighed 4.72 Gm. (85.5% of theory). The overall yield based on α-iodo diglyceride was 59%; m.p. 164–165°. At room temperature this waxy substance was highly soluble in chloroform, absolute ethanol, ethyl acetate, acetic acid, and benzene, moderately soluble in methanol, and insoluble in acetone, ether, and petroleum ether. It readily formed an emulsion in water.

Anal.—Calcd. for $C_{26}H_{50}NO_{10}P$ (576.65): C, 55.01; H, 8.88; N, 2.47; P, 5.46. Found: C, 55.19; H, 8.75; N, 2.46; P, 5.43.

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Determination of the Oil/Water Distribution Coefficients of Glyceryl Trinitrate and Two Similar Nitrate Esters

By FREDERICK K. BELL, JOHN J. O'NEILL, and RAYMOND M. BURGISON

The oil/water distribution coefficients of glyceryl trinitrate, chloroglyceryl dinitrate (1-chloro-2,3-propanediol dinitrate), and ethylglyceryl trinitrate (1,2,3-pentanetriol trinitrate) are determined. The analytical method employed (described in detail) is based on the reduction of a portion of the nitrate of each ester to the nitrite upon alkaline hydrolysis. This was determined by diazotization of sulfanilic acid and subsequent coupling with N-(1-naphthyl)-ethylenediamine and measuring the color spectrophotometrically.

In connection with certain pharmacologic studies being conducted in this laboratory, information was desired concerning the oil/water distribution coefficient of glyceryl trinitrate and two closely related compounds of similar structure, *viz.*, chloroglyceryl dinitrate (1-chloro-2,3-propanediol dinitrate) and ethylglyceryl trinitrate (1,2,3-pentanetriol trinitrate). Since the water solubility of these substances is very low, the problem was to develop a method of estimation applicable to dilute aqueous solutions.

Under similar conditions, numerous workers have employed (in various modifications) the well known procedure of alkaline hydrolysis of glyceryl trinitrate resulting in the formation of nitrite which can be determined spectrophotometrically with a high degree of sensitivity through the customary diazotization and coupling procedure. We found this general method of analysis for glyceryl trinitrate, used successfully by Frauch and Burgin (1), applicable to the other two nitrate esters. This communication describes the method we have developed and its application to the determination of the desired oil/water distribution coefficients.

EXPERIMENTAL

Materials.—The three nitrate esters used for study were prepared in this laboratory.

1-Chloro-2,3-propanediol dinitrate was prepared according to the method of Henry (2). It assayed 99.2%.

Glyceryl trinitrate was prepared by nitration of C.P. glycerin with mixed sulfuric-nitric acids (3). It was purified by repeated washings with water and very dilute sodium bicarbonate solution to remove soluble impurities and traces of nitration acids, then dried over anhydrous sodium sulfate. It assayed 99.0%.

1,2,3-Pentanetriol trinitrate was obtained by the nitration of 1,2,3-pentanetriol ("ethyl glycerine") with a mixture of equal volumes of red fuming nitric (d. 1.60) and concentrated sulfuric acid at −10 to 0°. One-hundred and fifty milliliters of mixed acid was used to nitrate 20 ml. of the triol. The crude nitrate, obtained as a blue oil upon pouring the nitration mixture into 1 L. of finely crushed ice, was purified by repeated washings with 500-ml. portions of ice water until the oil was almost colorless and the washings nearly neutral. The purified oil was dried over anhydrous sodium sulfate. Yield: 8.6 Gm. d_{20}^{20} 1.4377, nD^{20} 1.4570. It is miscible with alcohol and acetone in all proportions and very slightly soluble in water. Analyses, adapting the U.S.P. XV–Devarda's alloy reduction procedure for glyceryl trinitrate, indicated a purity of 101.5%.

Reagents and Apparatus.—Wherever possible reagent grade chemicals were used. The following aqueous solutions are required: sodium hydroxide (2 *N*), hydrochloric acid (4 *N*), sulfanilic acid (2 mg./ml.), and N-(1-naphthyl)-ethylenediamine dihydrochloride (1 mg./ml.). This latter solution was found to be sufficiently stable over a period of some weeks when stored in an amber glass bottle. The oil used for the distribution experiments was Wesson corn oil.

The complete reaction, including hydrolysis and subsequent diazotization, was carried out conveniently in 15-ml. graduated glass-stoppered Pyrex centrifuge tubes. The colorimetric determinations were made with a Beckman model DU spectrophotometer using absorption cells having a light path of 1 cm. An efficient shaking device is required which can be regulated to avoid the formation of stubborn oil/water emulsions.

Procedure.—Standard solutions of each of the three esters, which are required for the establishment of standard curves, were prepared in the following manner: approximately 50 mg. of the nitrate ester was accurately weighed into a 50-ml. volumetric flask. One milliliter of absolute methanol was added and thoroughly mixed with the ester. Forty milliliters of warm water was added, immediately followed by vigorous shaking until the solution was complete. After cooling to room temperature, water was added to the mark followed by thorough mixing. These standard solutions were diluted quantitatively for use. A concentration of 5 mcg./ml. is satisfactory.

Triplicate aliquots of this diluted standard of 0.5, 1.0, 1.5, 2.0, and 2.5 ml. were transferred to the graduated centrifuge tubes; water was added to the

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4-ml. mark. Three tubes containing 4 ml. of water served as controls. After the addition of 1 ml. of 2 *N* sodium hydroxide, mixing was accomplished by the inversion of the glass-stoppered tube. After the removal of the stoppers, the tubes were placed in a boiling water bath. It is important at this step to employ a rack of sufficient size to hold all the tubes of a given experiment so that they can be immersed in the boiling water and removed simultaneously. After a period of 10 minutes in the boiling water, the tubes are removed and (after cooling to room temperature) the contents of each tube were treated with the following reagents, respectively, (with mixing after each addition by inversion of the glass-stoppered tube): (*a*) 1 ml. 4 *N* hydrochloric acid, (*b*) 1 ml. sulfanilic acid solution, (*c*) 1 ml. N-(1-naphthyl)-ethylenediamine solution plus water to the 10-ml. mark. After standing 10 minutes, the absorbance of the solution in each tube was measured at 548 mμ with a slit width of approximately 0.03 mm. Distilled water was used as the reference standard. The corrected triplicate absorbance values were averaged and plotted against the concentration. Typical standard recovery curves for the three esters are shown in Fig. 1. A linear relation is apparent for each ester and the reproducibility was satisfactory.

For the determination of the oil/water distribution coefficients, the following procedure was carried out for each of the nitrate esters: approximately 25 ml. of corn oil was transferred to a tared 250-ml. glass-stoppered Erlenmeyer flask and accurately weighed. A second accurate weighing was made after the addition of a suitable amount of the ester (0.1 to 0.3 Gm.). After gentle rotation of the flask for some seconds, exactly 25 ml. of distilled water was added. A similar flask containing 25 ml. of corn oil and 25 ml. of distilled water served as a control. The flasks were stoppered and shaken for 1 hour at room temperature. A good separation of the two phases was obtained after standing 15 minutes. Then a portion of the aqueous layer was removed and passed through No. 2 Whatman filter paper. Aliquots of 0.1 or 0.2 ml. of these filtrates were then analyzed by the same procedure employed for the preparation of the standard curves. A summary of the analytical data is shown in Table I. The final values obtained for the oil/water distribution coefficients are: 109 ± 3 for glyceryl trinitrate, 146 ± 3 for chloroglyceryl dinitrate, and 97 ± 2 for ethylglyceryl trinitrate.

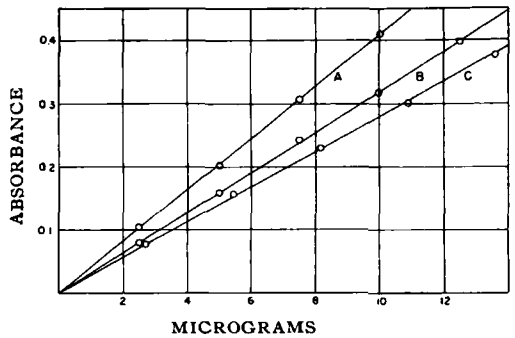

Fig. 1.—Typical standard recovery curves for the three esters: A, glyceryl trinitrate; B, chloroglyceryl dinitrate; C, ethylglyceryl trinitrate.

TABLE I.—SUMMARY OF EXPERIMENTAL DATA FOR THE DETERMINATION OF OIL/WATER DISTRIBUTION COEFFICIENTS

Ester	Expt.	Total Ester, Gm.	Ester in H_2O Layer, mg.	Dist. Coeff. Oil/H_2O
Glyceryl trinitrate	1	0.1676	1.70	110
	2	0.2267	2.33	110
	3	0.2829	2.84	108
	4	0.1191	1.25	106
	5	0.2412	2.50	112
	6	0.1619	1.65	109
				Av. 109 ± 3
Chloroglyceryl dinitrate	1	0.1266	0.95	147
	2	0.2015	1.51	149
	3	0.2530	1.90	148
	4	0.1690	1.32	143
	5	0.2249	1.72	145
	6	0.2338	1.85	143
				Av. 146 ± 3
Ethylglyceryl trinitrate	1	0.1778	2.00	98
	2	0.1057	1.25	95
	3	0.1419	1.55	98
				Av. 97 ± 2

DISCUSSION

According to Hay (4), the saponification of glyceryl trinitrate is represented by

$$C_3H_5(ONO_2)_3 + 5\ KOH = KNO_3 + 2\ KNO_2 + HCOOK + CH_3COOK + 3\ H_2O$$

indicating a stoichiometry of reaction not supported by analytical data. According to Frauch and Burgin (1) the ratio of 2 moles of nitrite per mole of ester is usually lower and varies with the experimental conditions. The decomposition of nitrate esters is undoubtedly more complex, involving competition between hydrolytic reactions yielding nitrate, and internal oxidation-reduction (5) accounting for the formation of reduction products (nitrite and hydroxylamine) (6) and various oxidation products indicated in Hay's equation.

In this connection we have replotted the curves from Fig. 1 on a molar basis (as shown in Fig. 2) and have included for comparison a standard curve for sodium nitrite prepared from a standard solution of a reagent grade of this salt. It may be seen that the base catalyzed reaction of glyceryl trinitrate by

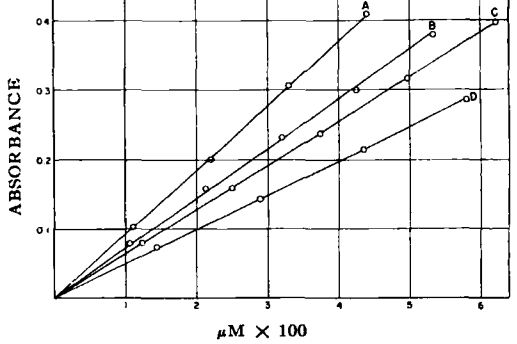

Fig. 2.—Typical standard recovery curves plotted on a molar basis: A, glyceryl trinitrate; B, ethylglyceryl trinitrate; C, chloroglyceryl dinitrate; D, sodium nitrite.

our procedure yields a nitrite:ester ratio appreciably lower than the value predicted by Hay.

If we apply the same general considerations to reactions involving the chloroglyceryl dinitrate and ethylglyceryl trinitrate and calculate ratios, we obtain an appreciably lower value in nitrite yield in the case of ethylglyceryl trinitrate. This suggests that the presence of an ethyl group has increased the stability of the molecule to oxidation-reduction or has favored reactions not resulting in nitrite formation. In the case of chloroglyceryl dinitrate having no nitrate group on carbon-1, an even higher degree of stability is indicated.

It appears that in each of the three nitrate esters one nitrate group is readily reduced and a second nitrate is reduced more slowly (or less completely). In spite of this apparent lack of stoichiometry, the highly consistent results obtained from repeated trials indicate that with reasonable care in the control of experimental conditions employing standard recovery curves, a satisfactory analytical procedure can be devised.

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Investigation of the Stereochemistry of Cycloheximide and Its Degradation Products

By HOWARD J. SCHAEFFER and VIJAY K. JAIN

Cycloheximide tosylate has been shown to undergo elimination of *p*-toluenesulfonic acid to give a new anhydrocycloheximide. By a series of reactions this new anhydrocycloheximide has been converted into a dideoxycycloheximide which was shown to be optically inactive. This observation establishes the cis-orientation of the methyl groups in the new dideoxycycloheximide. The series of reactions employed and the stereochemistry of the products obtained are discussed.

THE DETERMINATION of the stereochemistry of the glutarimide antibiotics has been under investigation recently in a number of laboratories (1–4, 7). On the basis of these studies, it has been demonstrated both by chemical degradation (7) and by thermal degradation (1) that the two methyl groups in cycloheximide are oriented in a *trans* manner. We have now observed certain other degradative and isomerization reactions on cycloheximide which bear on its stereochemistry.

Treatment of cycloheximide (I) with *p*-toluenesulfonyl chloride in pyridine gave a good yield of the corresponding tosylate (II). When an attempt was made to displace the tosylate group in II with iodide ion using acetone as the solvent, only unchanged starting material was recovered. Therefore, II was treated with sodium iodide in dimethylformamide; the product isolated from this reaction was not the corresponding iodide, but rather an iodine-free compound which exhibited ultraviolet absorption at 235 mμ (ϵ, 9480). On the basis of its elemental analysis, infrared and ultraviolet spectra, and its further reactions, this compound has been shown to be *epi*-anhydrocycloheximide (III).[1] Subsequently, it was learned that this interesting elimination and rearrangement reaction occurred merely by heating II in dimethylformamide. However, if the reaction is carried out in the presence of one equivalent of sodium bicarbonate, elimination of *p*-toluenesulfonic acid occurs with the formation of anhydrocycloheximide (IV)—identical with anhydrocycloheximide prepared by known procedures (5).

Catalytic hydrogenation of *epi*-anhydrocycloheximide (III) with a palladium-on-charcoal catalyst gave *epi*-deoxycycloheximide (V) which upon further hydrogenation with a platinum catalyst gave *epi*-dihydrodeoxycycloheximide (VI). Compound VI could also be prepared by the direct hydrogenation of III with a platinum catalyst. When VI was allowed to react with phosphorus tribromide in dioxane solution, the corresponding bromo compound VII was obtained which after reaction with zinc and acetic acid

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[1] In order to distinguish the degradation products which we have prepared in this research from the degradation products which have previously been prepared (5, 7), we have used the prefix *epi* for our new compounds. In each case, the *epi* compounds have been shown to be different from the previously prepared degradation products by comparing the infrared and ultraviolet spectra, melting points and mixture melting points, and by thin-layer chromatography.

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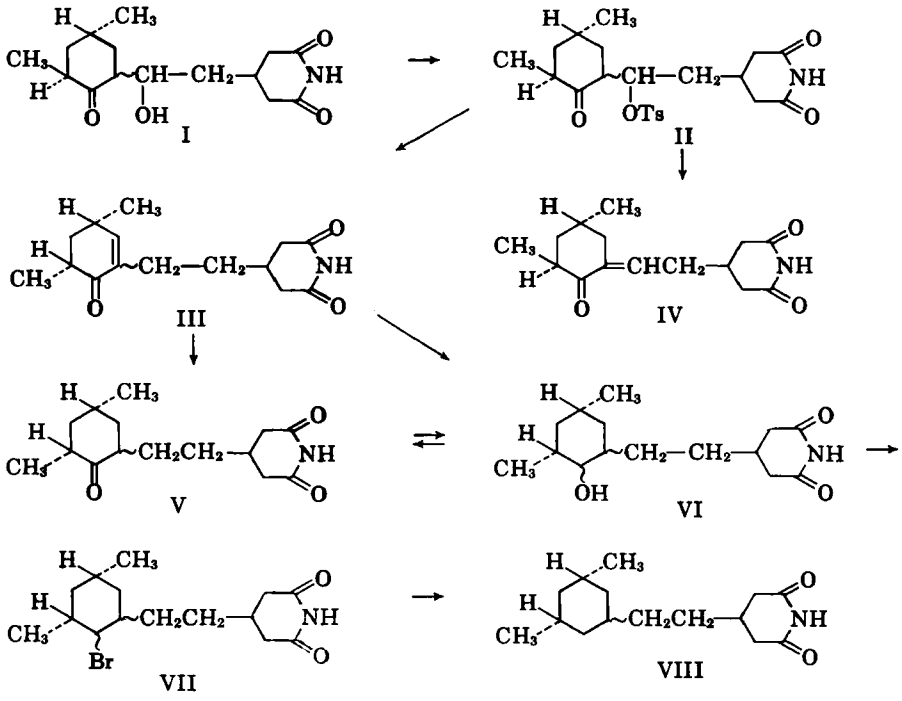

gave *epi*-dideoxycycloheximide (VIII). An attempt to determine the optical activity of VIII revealed that it was optically inactive. This observation establishes that the methyl groups in VIII are *cis* since only the *cis*-compound possesses a plane of symmetry and therefore must be optically inactive.

Since it is known that the methyl groups in I (1, 7), and therefore in II, are *trans*, it is now apparent that during the preparation of III from II—in addition to the rearrangement of the double bond from *exo* to *endo*—an isomerization of the methyl group alpha to the ketone occurred to give the thermodynamically stable *cis* isomer. When III is hydrogenated with a palladium-on-charcoal catalyst, a new *epi*-deoxycycloheximide (V) is obtained. Hydrogenation of V with a platinum catalyst gave a new *epi*-dihydrodeoxycycloheximide (VI). Oxidation of VI with chromium trioxide in glacial acetic acid gave a good yield of V. An attempted isomerization of V with *p*-toluenesulfonic acid in dimethylformamide resulted in the recovery (in good yield) of unchanged starting material. Therefore, these results make it improbable that isomerization occurred during the hydrogenation of III. Since it is difficult to visualize an isomerization occurring in subsequent reactions, the assignment of the *cis*-methyl groups in III, V, VI, and VII is most probably justified.

By a series of reactions similar to those used in this work, anhydrocycloheximide (IV) was converted into a dideoxycycloheximide which was optically active (7). The observation that the dideoxycycloheximide was optically active established that the methyl groups were *trans;* therefore the methyl groups in cycloheximide (I) were also *trans*. Because the compounds obtained in this investigation are stereoisomeric with the compounds obtained in the previous series, we believe that our assignment of the *trans* configuration of methyl groups in the previous series is supported by the present work.

EXPERIMENTAL[2]

Cycloheximide Tosylate (II).—A solution of cycloheximide (3.96 Gm., 14.1 mmoles) in pyridine (15 ml.) was cooled in an ice bath for 30 minutes and *p*-toluenesulfonyl chloride (2.86 Gm., 15.4 mmoles) was added. The reaction mixture was allowed to stand overnight at room temperature and then was poured onto ice. After the ice had melted, the crystals were collected by filtration, washed with water, and then dried *in vacuo* at room temperature. Yield, 4.45 Gm. (71.7%) of cycloheximide tosylate, m.p. 115°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3200 and 3100 (NH), 1710 and 1700 (C = 0), 1600 (phenyl). One recrystallization of the crude material from benzene gave the analytical sample, m.p. 114°. The compound decomposed on standing at room temperature. This compound has recently been prepared by Okuda, who reported a m.p. of 100.5 to 101.5° (6).

[2] The infrared spectra were determined on a Perkin-Elmer model 137 spectrophotometer; the ultraviolet spectra were determined on a Perkin-Elmer model 4000A spectrophotometer. The melting points were determined on a Kofler Heizbank and are corrected.

Anal.[3]—Calcd. for $C_{22}H_{29}NO_6S$: C, 60.66; H, 6.71; N, 3.21; S, 7.36. Found: C, 60.19; H, 6.34; N, 3.13; S, 6.90.

***epi*-Anhydrocycloheximide (III).**—A solution of cycloheximide tosylate (3.6 Gm., 8.4 mmoles) in 12 ml. dimethylformamide was heated under reflux for 40 minutes. The reaction mixture was allowed to cool to room temperature and then poured onto ice. After the ice had melted, the crystals were collected by filtration, and washed with water. Yield, 1.3 Gm. (59%) of *epi*-anhydrocycloheximide, m.p. 83–84°. On recrystallization from ethanol and water, the crude material gave the analytical sample, m.p. 85–86°. $\lambda_{max.}^{EtOH}$ ($\epsilon \times 10^{-3}$) 235 mμ (9.48), $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3200 and 3100 (NH), 1740 and 1690 (C = 0).

Anal.—Calcd. for $C_{15}H_{21}NO_3$: C, 68.41; H, 8.04; N, 5.32; Found: C, 67.96; H, 7.91; N, 5.38.

Anhydrocycloheximide (IV).—A mixture of cycloheximide tosylate (1.0 Gm., 2.3 mmoles) and sodium bicarbonate (0.4 Gm., 4.6 mmoles) in dimethylformamide (15 ml.) was heated under reflux for 20 minutes. The reaction mixture was allowed to cool to room temperature and then poured onto ice. After the ice had melted, the crystals were collected by filtration and washed with water. Yield, 0.36 Gm. (60%) of anhydrocycloheximide, m.p. 125–127°. On recrystallization from benzene and hexane, the crude material gave the pure sample, m.p. 132–134°; mixed m.p. with authentic sample of anhydrocycloheximide 130 to 133.5° (5). $\lambda_{max.}^{EtOH}$ 240 mμ (ϵ = 8140); $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3200 and 3100 (NH), 1710 and 1690 (C = 0), 1610 (C = C).

***epi*-Deoxycycloheximide (V).**—A solution of 0.20 Gm. (0.76 mmole) of *epi*-anhydrocycloheximide in 200 ml. of absolute alcohol was added to 150 mg. of 5% palladium-on-charcoal catalyst which had been wetted with a few drops of water. The mixture was hydrogenated at room temperature and at an initial pressure of 40.7 p.s.i. until no more hydrogen was absorbed. The solution was filtered, and the solvent was removed *in vacuo*. The residue was crystallized from ethanol and water and gave 0.13 Gm. (65%) of *epi*-deoxycycloheximide, m.p. 151–152°. Two recrystallizations of the crude product from ethanol and water gave the analytical sample, m.p. 151–152°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3100 and 3200 (NH), 1690, 1700, and 1730 (C = 0). $[\alpha]_D^{24}$ = −4.1 (c = 4.8 $CHCl_3$).

Anal.—Calcd. for $C_{15}H_{23}NO_3$: C, 67.89; H, 8.74; N, 5.27. Found: C, 67.93; H, 8.63; N, 5.34.

***epi*-Dihydrodeoxycycloheximide (VI).**—A solution of 7.39 Gm. (28.0 mmoles) of *epi*-anhydrocycloheximide in 200 ml. of glacial acetic acid was added to a pre-reduced platinum dioxide catalyst (1.50 Gm.). The mixture was hydrogenated at room temperature at 59.8 p.s.i. Reduction was complete after 55.2 mmoles of hydrogen had been absorbed. After filtration, the solvent was removed *in vacuo*, and the semisolid residue on crystallization from benzene and hexane gave 2.22 Gm. (29.6%) of *epi*-dihydrodeoxycycloheximide, m.p. 137–138°. Two recrystallizations from methanol and water gave the analytical sample, m.p. 140–141°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3500 (OH), 3240 and 3110 (NH), 1690 and 1710 (C = 0). $[\alpha]_D^{23}$ = −0.309 (c = 3.2 acetone).

Anal.—Calcd. for $C_{15}H_{25}NO_3$: C, 67.38; H, 9.42; N, 5.24. Found: C, 67.16; H, 9.54; N, 5.15.

Hydrogenation of *epi*-Deoxycycloheximide.—A solution of 0.21 Gm. (0.80 mmole) of *epi*-deoxycycloheximide in 200 ml. glacial acetic acid was added to 75 mg. of pre-reduced platinum dioxide catalyst. The mixture was hydrogenated at room temperature until the absorption of hydrogen stopped. After filtration, the solvent was removed *in vacuo*, and the semisolid residue on crystallization from methanol and water gave 0.15 Gm. (71.3%) of *epi*-dihydrodeoxycycloheximide, m.p. 140.5 to 142°, mixed m.p. with an authentic sample of *epi*-dihydrodeoxycycloheximide 140.5–141.5°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3500 (OH); 3200 and 3100 (NH), 1690 and 1710 (C = 0).

Dihydrodeoxycycloheximide Bromide (VII).—A solution of *epi*-dihydrodeoxycycloheximide (0.28 Gm., 1.1 mmole) in *p*-dioxane (5 ml.) was cooled in an ice bath for 15 minutes; a solution of phosphorus tribromide in *p*-dioxane (5 ml.) was added. The reaction mixture was allowed to stand overnight at room temperature and then concentrated *in vacuo*. The colorless residue after crystallization from benzene and hexane gave 0.10 Gm. (27%) of *epi*-dihydrodeoxycycloheximide bromide, m.p. 142–144°. On recrystallization from benzene and hexane, the crude material gave the analytical sample, m.p. 151 to 151.5°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3100 and 3200 (NH), 1690, and 1740 (C = 0).

Anal.—Calcd. for $C_{15}H_{24}NO_2Br$: C, 54.54; H, 7.32; N, 4.24; Br, 24.19. Found: C, 54.33: H, 7.15; N, 4.25; Br, 24.09.

***epi*-Dideoxycycloheximide (VIII).**—Zinc dust (0.85 Gm.) was added to a solution of *epi*-dihydrodeoxycycloheximide bromide (0.58 Gm., 1.75 mmoles) in 10.6 ml. of glacial acetic acid; the reaction mixture was stirred for 2 hours at 80°. After filtration, water was added to the filtrate which caused crystallization of the crude product. Yield, 0.34 Gm. (77.2%) of *epi*-dideoxycycloheximide, m.p. 120°. Two recrystallizations from methanol and water gave the analytical sample, m.p. 124–126°. $\bar{\nu}$ in cm.$^{-1}$ (KBr): 3290 and 3180 (NH); 1740 and 1690 (C = 0). $[\alpha]_D^{24}$ = 0.0 (c = 1.496 acetone).

Anal.—Calcd. for $C_{15}H_{25}NO_2$: C, 71.67; H, 10.02; N, 5.57. Found: C, 71.51; H, 9.85; N, 5.69.

Oxidation of *epi*-Dihydrodeoxycycloheximide.—To a solution of 0.21 Gm. (0.78 mmoles) of *epi*-dihydrodeoxycycloheximide in 5.1 ml. of glacial acetic acid was added a solution of chromic acid (0.69 Gm.) in 0.75 ml. of water. The reaction solution was heated on steam bath for 10 minutes, then was allowed to stand for 3 hours at room temperature. After dilution with 20 ml. of water, the solution was extracted with chloroform (four 20-ml. portions). The organic extract was washed with a saturated solution of sodium bicarbonate (three 25-ml. portions), water (30 ml.), dried with anhydrous magnesium sulfate, and then filtered. The volatile materials were removed *in vacuo*, and the semisolid residue on crystallization from ethanol and water gave 0.13 Gm. (66.7%) of *epi*-deoxy-

[3] The analyses in this paper were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

cycloheximide, m.p. 151–153°; mixed m.p. 150 to 151.5° with an authentic sample of *epi*-deoxycycloheximide. $\tilde{\nu}$ in cm.$^{-1}$ (KBr): 3200 and 3100 (NH), 1730, 1700, and 1680 (C = 0). The *epi*-deoxycycloheximide was further identified by thin-layer chromatography on silica gel when compared with the authentic sample of *epi*-deoxycycloheximide (R_f = 0.15, in chloroform containing 1% methanol).

Attempted Isomerization of *epi*-Deoxycycloheximide.—A solution of 0.10 Gm. (0.38 mmole) of *epi*-deoxycycloheximide and *p*-toluenesulfonic acid (0.08 Gm., 0.41 mmole) in 2.0 ml. of dimethylformamide was refluxed for 40 minutes. The reaction mixture was allowed to cool to room temperature and then poured onto ice. After the ice had melted, the crystals were collected by filtration and dried *in vacuo;* yield, 0.07 Gm. of *epi*-deoxycycloheximide, m.p. 140°. On recrystallization from ethanol and water, the crude material gave the pure sample, m.p. 149.5 to 152°; mixed m.p. 150–152° with the authentic sample of *epi*-deoxycycloheximide. $\tilde{\nu}$ in cm.$^{-1}$ (KBr): 3200 and 3100 (NH), 1730, 1700, and 1680 (C = 0). The *epi*-deoxycycloheximide was further identified by thin-layer chromatography on silica gel when compared with the authentic sample of *epi*-deoxycycloheximide (R_f = 0.15 chloroform containing 1% methanol).

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Photometric Determination of Pentylenetetrazol in Pharmaceutical Preparations

By ROBERT A. DAOUST

Pentylenetetrazol is quantitatively precipitated from aqueous solution by cuprous chloride reagent. The pentylenetetrazol–cuprous chloride complex is dissolved in concentrated nitric acid; the solution is adjusted to pH 4 with acetate buffer and sodium hydroxide solution, and the deep blue color of the copper complex developed with tetraethylenepentamine is determined photometrically at 580 mμ. The method can be applied to samples containing from 2–10 mg. pentylenetetrazol and furnishes a rapid means of determining small amounts of the drug in the presence of other ingredients in pharmaceutical preparations.

PENTYLENETETRAZOL, a white, odorless, crystalline drug with a slightly pungent bitter taste is applied extensively as a central nervous system stimulant in the treatment of schizophrenia in shock therapy of psychoses and as an analeptic in narcotic poisoning.

This paper describes a method for the photometric determination of pentylenetetrazol in pharmaceutical liquids and tablets; the method can be performed rapidly and simply on small amounts of samples suitable for pharmaceutical control work. A gravimetric method which is determined by salting the drug from its aqueous solution with ammonium sulfate, followed by extraction with carbon tetrachloride has been officially adopted by "The National Formulary" (1, 2). Another gravimetric method involving precipitation with phosphotungstic acid is favored by some workers (3, 4). Numerous procedures have been reported for the assay of pentylenetetrazol: refractometric (5, 6), indirect polarographic (7), complexometric (8–10), colorimetric (11, 12), and volumetric and potentiometric methods (12).

The principle of the method proposed involves the precipitation of pentylenetetrazol from its aqueous solution with an excess of cuprous chloride reagent to give an insoluble, silver-white, crystalline, double salt complex which is then dissolved in concentrated nitric acid. The acid decomposes the pentylenetetrazol–cuprous chloride complex into its components and simultaneously oxidizes the cuprous salt to the cupric form. The acid solution is adjusted to pH 4 with acetate buffer and sodium hydroxide solution, and the absorbance of the deep blue color of the copper complex developed with tetraethylenepentamine

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Pentylenetetrazol is quantitatively precipitated from aqueous solution by cuprous chloride reagent. The pentylenetetrazol–cuprous chloride complex is dissolved in concentrated nitric acid; the solution is adjusted to pH 4 with acetate buffer and sodium hydroxide solution, and the deep blue color of the copper complex developed with tetraethylenepentamine is determined photometrically at 580 mμ. The method can be applied to samples containing from 2–10 mg. pentylenetetrazol and furnishes a rapid means of determining small amounts of the drug in the presence of other ingredients in pharmaceutical preparations.

PENTYLENETETRAZOL, a white, odorless, crystalline drug with a slightly pungent bitter taste is applied extensively as a central nervous system stimulant in the treatment of schizophrenia in shock therapy of psychoses and as an analeptic in narcotic poisoning.

This paper describes a method for the photometric determination of pentylenetetrazol in pharmaceutical liquids and tablets; the method can be performed rapidly and simply on small amounts of samples suitable for pharmaceutical control work. A gravimetric method which is determined by salting the drug from its aqueous solution with ammonium sulfate, followed by extraction with carbon tetrachloride has been officially adopted by "The National Formulary" (1, 2). Another gravimetric method involving precipitation with phosphotungstic acid is favored by some workers (3, 4). Numerous procedures have been reported for the assay of pentylenetetrazol: refractometric (5, 6), indirect polarographic (7), complexometric (8–10), colorimetric (11, 12), and volumetric and potentiometric methods (12).

The principle of the method proposed involves the precipitation of pentylenetetrazol from its aqueous solution with an excess of cuprous chloride reagent to give an insoluble, silver-white, crystalline, double salt complex which is then dissolved in concentrated nitric acid. The acid decomposes the pentylenetetrazol–cuprous chloride complex into its components and simultaneously oxidizes the cuprous salt to the cupric form. The acid solution is adjusted to pH 4 with acetate buffer and sodium hydroxide solution, and the absorbance of the deep blue color of the copper complex developed with tetraethylenepentamine

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(13) is measured at 580 mμ with a suitable spectrophotometer.

EXPERIMENTAL

Reagents.—*Dister's Reagent* (14). A 1.250 Gm. quantity of cuprous chloride (reagent grade) is dissolved in distilled water to make 100 ml. of an aqueous solution containing 10 Gm. of ammonium chloride U.S.P. and 1 Gm. of sodium *meta*-bisulfite (reagent grade). This solution, if kept in a tightly stoppered amber bottle in a refrigerator immediately after use, is stable for several weeks.

Pentylenetetrazol Standard Solution. One-hundred milligrams of dried crystalline pentylenetetrazol N.F. is dissolved in about 50 ml. distilled water and the solution is diluted to the mark of a 100-ml. glass-stoppered volumetric flask. Each milliliter of this solution contains 1.0 mg. pentylenetetrazol.

Acetate Buffer Solution (15). A 16.6 Gm. quantity of anhydrous sodium acetate (reagent grade) is dissolved in about 100 ml. distilled water; 24 ml. glacial acetic acid is added and the mixture diluted to 200 ml.

Sodium Hydroxide Solution, 6% (w/v). Twelve grams of sodium hydroxide pellets (reagent grade) is dissolved in distilled water to make 200 ml. of solution.

Tetraethylenepentamine Solution, 2% (w/v). Four milliliters of tetraethylenepentamine (technical grade) is mixed with distilled water to make 200 ml. of solution.

TABLE I.—RELATION BETWEEN CONCENTRATION AND ABSORBANCE OF PENTYLENETETRAZOL

Concn., mg./50 ml.	Absorbance, S.D.
2	0.095 ± 0.008
4	0.177 ± 0.006
5	0.228 ± 0.004
6	0.267 ± 0.007
8	0.356 ± 0.011
10	0.444 ± 0.005

Dilute Acetic Acid Solution, 1% (v/v). One milliliter of glacial acetic acid is mixed with distilled water to make 100 ml. of solution.

Concentrated Nitric Acid (reagent grade).

Preparation of the Calibration Graph.—Aliquots of 2, 4, 6, 8, and 10 ml. of the standard solution of pentylenetetrazol are pipeted in order into each of five 50-ml. Erlenmeyer flasks. Each of the first four solutions is diluted to 10 ml. with distilled water, and 2 ml. of Dister's reagent is added to each of the five solutions. The flasks are stoppered and placed in an ice bath for 20 minutes with swirling a few times at 5-minute intervals to insure complete precipitation.

The precipitate is then filtered with suction through a 30-ml. sintered Pyrex glass filter funnel of medium porosity attached to a 500-ml. Pyrex glass suction flask; the filtrate, which is collected in a 18 x 150-mm. test tube placed inside of the flask, is rejected. The precipitate is washed twice with 5-ml. portions of cold dilute 1% acetic acid solution and the washings rejected.

The residue is dissolved by adding 1 ml. of concentrated nitric acid directly onto the sintered-glass funnel using a safety pipet or 50-ml. glass syringe. The filtrate is collected in the same manner in the test tube. The walls and plate of the glass funnel are washed with about 5 ml. of cold distilled water and aspirated through into the filtrate. The test tube (containing the filtrate) is carefully lifted out of the flask by a wire bent into a hook placed around the test tube. The filtrate is then poured into a 50-ml. glass-stoppered volumetric flask by means of a glass buret funnel. The washing is repeated with another 5 ml. portion and this is poured into the flask also. Ten milliliters each of the acetate buffer, 6% aqueous sodium hydroxide solution, and 2% aqueous tetraethylenepentamine solution is pipeted in succession into the acid solution; diluted to the mark with distilled water, stoppered, and mixed well. A blank solution is prepared by pipeting in succession into another 50-ml. glass-stoppered volumetric flask (containing a little distilled water) 1 ml. of concentrated nitric acid, 10

TABLE II.—RESULTS OF ASSAYS FOR PENTYLENETETRAZOL ON VARIOUS PHARMACEUTICAL LIQUIDS (100 MG./5 ML.) AND TABLETS (100 MG./TABLET)

Vita-Metrazol[a]		Metrazol-Liquidum[b]	
Absorbance of Sample[e]	Recovery, %	Absorbance of Sample[f]	Recovery, %
0.240	100.0	0.235	100.0
0.230	95.8	0.235	100.0
0.235	97.9	0.230	97.8
0.235	97.9	0.230	97.8
0.238	99.1	0.230	97.8
Mean	98.2		98.7
S.D.	±1.6		±1.2

Nico-Metrazol[c]		Metrazol Tablets[d]		
Absorbance of Sample[g]	Recovery, %	Absorbance of Sample[h]	Wt. Sample, Gm.	Recovery, %
0.230	102.2	0.225	0.3368	97.4
0.230	102.2	0.235	0.3359	101.9
0.230	102.2	0.235	0.3362	101.9
0.225	100.0	0.240	0.3374	103.7
0.235	104.4	0.225	0.3368	97.4
Mean	102.2			100.5
S.D.	±1.6			±2.9

[a] Each 5 ml. contains 100 mg. Metrazol, 10 mg. niacinamide, 1 mg. pyridoxine hydrochloride, 1.4 mg. riboflavin sodium phosphate, and 2 mg. *d*-panthenol dissolved in 100 ml. of 15% ethanol. [b] Each 5 ml. contains 100 mg. Metrazol and 1 mg. thiamine hydrochloride in 15% ethanol. [c] Each 5 ml. contains 100 mg. Metrazol and 50 mg. nicotinic acid in 15% ethanol. [d] Based on an average tablet weight of 0.3353 Gm. [e] Absorbance of standard of 5 mg. concentration = 0.240. [f] Absorbance of standard of 5 mg. concentration = 0.235. [g] Absorbance of standard of 5 mg. concentration = 0.225. [h] Absorbance of standard of 5 mg. concentration = 0.230.

ml. each of acetate buffer, 6% sodium hydroxide solution, and 2% tetraethylenepentamine solution; the solution is diluted to the mark with distilled water, stoppered, and mixed well. The zero absorbance of the Beckman spectrophotometer, model DB, is set with this solution.

After allowing the solutions to stand for 10 minutes, the absorbance of the deep blue copper tetraethylenepentamine complex is measured at its maximum absorption, 580 mμ. The calibration graph of pentylenetetrazol, plotted on a linear scale at 2-mg. increments in the range from 2–10 mg., indicated that the method obeys Beer's law with good agreement. It is a straight line which passes through the origin.

Table I shows the relation between the absorbance and concentration of the standard solutions of pentylenetetrazol (which is directly proportional to the copper tetraethylenepentamine complex). Each value of the absorbance is the mean of ten determinations.

Procedure for Tablets.—The average tablet weight is ascertained from the weight of 20 tablets weighed accurately. Transfer a sample of the pulverized tablets, containing approximately 100 mg. pentylenetetrazol, into a 100-ml. glass-stoppered volumetric flask, dissolve in distilled water, fill up to the mark, stopper, mix well, and filter through paper, rejecting the first portion of the filtrate. Pipet 5 ml. of the filtrate, containing approx. 5 mg. pentylenetetrazol, into a 50-ml. Erlenmeyer flask and add 5 ml. of distilled water and 2 ml. of Dister's reagent. Stopper the flask, place in an ice bath, and follow the procedure described for the *Preparation of the Calibration Graph*. The amount of pentylenetetrazol present can be determined from the graph, or comparison with the absorbance of a solution obtained by similar treatment of 5 ml. of the *Standard Solution*.

Procedure for Liquids.—Pipet an aliquot of the liquid sample, containing approx. 100 mg. pentylenetetrazol, into a 100-ml. glass-stoppered volumetric flask, dilute to the mark with distilled water, stopper, and mix well. Proceed by the directions given for the *Procedure for Tablets* beginning with "Pipet 5 ml. . . ." then calculate

$$\frac{\text{A sample}}{\text{A standard}} \times \frac{5\text{ mg.}}{50\text{ ml.}} \times \frac{100\text{ ml.}}{\text{wt. sample (Gm.)}} \times \frac{\text{average tablet wt.}}{1\text{ tablet}} \times \frac{50\text{ ml.}}{5\text{ ml.}} = \text{mg./tablet}$$

$$\frac{\text{A sample}}{\text{A standard}} \times \frac{5\text{ mg.}}{50\text{ ml.}} \times \frac{100\text{ ml.}}{\text{vol. sample (5 ml.)}} \times \frac{50\text{ ml.}}{5\text{ ml.}} = \text{mg./5 ml.}$$

Table II shows the results of assays carried out on pentylenetetrazol pharmaceutical preparations manufactured by the Knoll Pharmaceutical Co., Orange, N. J. The absorbance of a solution obtained by similar treatment of 5 ml. of the *Standard Solution* was measured at the same time as that of the sample.

DISCUSSION

It was observed that small concentrations up to 4 mg. pentylenetetrazol were not precipitated immediately with Dister's reagent at room temperature, except on longer standing with frequent swirling. For this reason, the solutions were chilled in an ice bath for 20 minutes with occasional swirling of the stoppered flasks.

It is evident that other active ingredients and materials usually present with pentylenetetrazol do not interfere with the photometric method.

Ephedrine sulfate is not precipitated by Dister's reagent, so it should not interfere if present with pentylenetetrazol. On the other hand, quinine sulfate and quinidine sulfate form yellow flocculent precipitates with Dister's reagent, so the procedure cannot be applied directly if these alkaloids are present, unless they are separated by chromatographic adsorption or some other technique. However, the alkaloids can be determined satisfactorily by this method, although the sensitivity is about half that of pentylenetetrazol.

Eight solutions of pentylenetetrazol can be assayed in about 2 hours by the proposed method.

SUMMARY

Pentylenetetrazol can be determined quantitatively in the presence of active ingredients and materials usually present in pharmaceutical liquids and tablets by precipitation from aqueous solution with cuprous chloride reagent. The double salt complex formed is decomposed into the copper ion which is determined photometrically. The method proposed is sensitive and gives reproducible results on small samples. Since the method is not specific for pentylenetetrazol, it may be possible to apply it satisfactorily to other substances which react quantitatively with the cuprous chloride reagent.

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Open-Chain Analogs of LSD II

Synthesis of Some 2-(3-Indolylethyl)- and 2-(3-Methyl-2-indolylethyl)piperidines

By CHARLES W. WHITTLE† and RAYMOND N. CASTLE

6-Methylpyridines substituted in the 3-position, which were quaternized with methyl or ethyl iodide, were condensed under Knoevenagel conditions with 3-indolealdehyde or with 3-methyl-2-indolealdehyde. The indolylethenylpyridinium iodides thus produced were hydrogenated to the corresponding indolylethylpiperidines. Conversion of the hydrogenation products into acid salts or into quaternary salts provided compounds for pharmacological testing. Some of these are open-chain analogs of LSD.

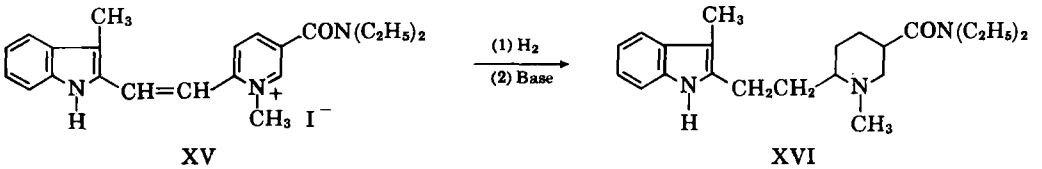
THE FIRST PAPER in this series (1) reported the synthesis of a number of 2-(3-indolylethenyl)-1-alkyl-5-substituted pyridinium iodides and some hydrogenation products. This series has now been extended and some of the compounds previously reported have been hydrogenated to the corresponding indolylethylpiperidines.

6-Methylnicotinoyl chloride was prepared by the action of oxalyl chloride on potassium 6-methylnicotinate according to the method of Wingfield (2). The acid chloride was not isolated but allowed to react directly with a secondary amine. In this manner 1-(6-methylnicotinoyl)-piperidine (I) and 4-(6-methylnicotinoyl)morpholine (II) were prepared. When I and II were allowed to react with methyl iodide, the quaternary salts were formed. When allowed to condense with 3-indolealdehyde, the quaternary salts of I and II gave 1-[6-(3-indolylethenyl)-nicotinoyl]piperidine methiodide (III) and 4-[6-(3-indolylethenyl)nicotinoyl]morpholine methiodide (IV).

Young (3) has shown that 3-indolealdehyde could be prepared in good yield by formylation of indole with a complex of N,N-dimethylformamide and phosphorus oxychloride. Using the same complex, Silverstein, Ryskiewiecz, and Willard (4) prepared 2-pyrrolealdehyde in 76% yield. Attempts to prepare 3-methyl-2-indolealdehyde (V) from skatole by the method of Young (3) gave 1-formyl-3-methylindole (VI) (55%) and V (20%). When the reaction temperature was changed from 5 to 60°, the yield of VI remained the same but the yield of V decreased. This is a contrast to the experience of Potokhin (5) who found that increased temperatures favored the formlyation of indole at position 3 over position 1.

Alessandri and Passerini (6) reported (without yields) that the 1-formyl-2-methylindole, when heated at 200° in the presence of zinc chloride, rearranged into 2-methyl-3-indolealdehyde. When VI was heated at 200° with zinc chloride no aldehyde was obtained. When it was heated at 250° a small amount of V was isolated. When V was allowed to react with 5-(N,N-diethylcarbamyl)-1,2-dimethylpyridinium iodide, 5 - (N,N - diethylcarbamyl) - 1 - methyl - 2-(3 - methyl - 2 - indolylethenyl)pyridinium iodide (XV) was obtained. When XV was hydrogenated, 5-(N,N-diethylcarbamyl)-1- methyl - 2 - (3 - methyl - 2 - indolylethyl)piperidine (XVI) was obtained as the hydriodide salt. This was readily converted into XVI by treatment with alkali.

Several indolylethenylpyridinium iodides were hydrogenated under high pressure to give the corresponding indolylethylpiperidines. These were converted into quaternary salts or into acid salts. The piperidine quaternary salts prepared were found to be less hygroscopic than the piperidine acid salts. Some of these transformations are shown.



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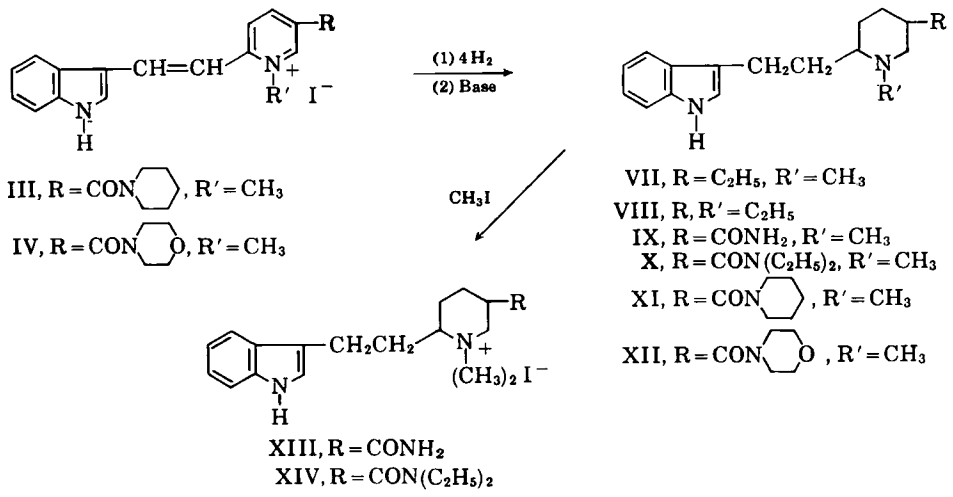

3-Methylindole-1-carboxylic acid, which occurs in the beet, has been prepared from skatole in 86% yield *via* the Grignard reaction.

The pharmacological screening of this group of compounds was discontinued because of the lack of interesting activity[1] when screened for mouse behavior, oxytocic, and antimicrobial activity.

EXPERIMENTAL

The melting points are uncorrected. The micro melting points (m.m.p.) were determined on a Kofler hot stage. Carbon, hydrogen, and nitrogen analyses were performed by Tanabe Seiyaku Co., Ltd., Tokyo, Japan.[2] Basic nitrogen was determined by nonaqueous titration with perchloric acid in acetic acid (7).

1-(6-Methylnicotinoyl)piperidine.—6-Methylnicotinoyl chloride was prepared by allowing 26.0 Gm. (0.148 mole) of potassium 6-methylnicotinate to react with 18.0 Gm. (0.148 mole) of oxalyl chloride in a manner previously described (2). Eighteen milliliters of dry piperidine was added to the crude acid chloride solution. The product was extracted as the hydrochloride into water, decolorized with charcoal, then made basic with sodium hydroxide solution. The water was removed by vacuum evaporation; the solid residue was extracted into ether by Soxhlet extraction. After evaporation of the dried (calcium chloride) ether solution, the compound was distilled at 143–146° at 0.25 mm. Thirteen and one-half grams (45%) of product was obtained, m.p. 63 to 64.5°.

Anal.—Calcd. for $C_{12}H_{16}N_2O$: C, 70.56; H, 7.90; N, 13.71. Found: C, 70.22; H, 7.79; N, 13.52.

4-(6-Methylnicotinoyl)morpholine.—Twenty milliliters of dry morpholine was added dropwise to a solution of 6-methylnicotinoyl chloride (prepared as above); the reaction mixture was refluxed for 30 minutes. The cooled reaction product was extracted into water as the hydrochloride and decolorized twice with charcoal. After neutralization with sodium hydroxide solution, the water was removed by vacuum evaporation and the organic material was extracted into hot benzene. Removal of the benzene left a green oil which distilled at 135–136° at 0.08 mm. The liquid was dissolved in anhydrous ether, and a stream of dry hydrogen chloride was bubbled through the ethereal solution until precipitation was complete. The salt was collected, washed with dry ether, dried, and then recrystallized twice from ethanol-ethyl acetate to give the hydrochloride, m.p. 232–233°.

Anal.—Calcd. for $C_{11}H_{14}N_2O_2 \cdot HCl$: C, 54.44; H, 6.23; N, 11.54. Found: C, 54.70; H, 6.33; N, 11.71.

The salt was decomposed with ammonium hydroxide solution. After vacuum evaporation, the compound was extracted into ether. Removal of the dried ether gave 17.0 Gm. (58%) of the purified liquid compound.

Anal.—Calcd. for $C_{11}H_{14}N_2O_2$: C, 63.89; H, 6.84; N, 13.58. Found: C, 64.02; H, 6.62; N, 13.38.

1-(6-Methylnicotinoyl)piperidine Methiodide.—A precipitate separated from the reaction mixture when 10.0 Gm. (0.049 mole) of 1-(6-methylnicotinoyl)piperidine and excess methyl iodide dissolved in 300 ml. of benzene were allowed to reflux for 20 hours. The solid product was collected, washed with benzene, and dried. Upon crystallization from ethanol, 15.8 Gm. (93%) of a white solid was obtained m.p. 185–186°.

Anal.—Calcd. for $C_{13}H_{19}IN_2O$: C, 45.10; H, 5.53; N, 8.09. Found: C, 44.89; H, 5.47; N, 8.09.

4-(6-Methylnicotinoyl)morpholine Methiodide.—When 15.0 Gm. (0.074 mole) of 4-(6-methylnicotinoyl)morpholine and excess methyl iodide were allowed to react as above, a precipitate separated from the reaction mixture. After two recrystallizations from ethanol, 19.0 Gm. (76%) of white platelets was obtained, m.p. 238–239°.

Anal.—Calcd. for $C_{12}H_{17}IN_2O_2$: C, 41.39; H, 4.92; N, 8.05. Found: C, 41.04; H, 5.04; N, 8.08.

3-Methylindole-1-carboxylic Acid.—To an ethereal solution of methyl magnesium iodide, prepared from 2.68 Gm. (0.11 mole) of magnesium

[1] The authors are indebted to the Lilly Research Laboratories for screening these compounds.
[2] The authors thank Dr. S. Yamada for this service.

turnings and 15.6 Gm. (0.11 mole) of methyl iodide, was added dropwise 13.1 Gm. (0.10 mole) of skatole in 25 ml. of dry ether. A heavy ether-insoluble oil resulted from the reaction to which an excess of dry ice was added in portions while the two phases were stirred vigorously with gentle heating. The complex was decomposed with dilute hydrochloric acid; the organic portion dissolved in ether and separated. The solid remaining after the removal of the ether was recrystallized twice from benzene. There was obtained 15.0 Gm. (86%) of 3-methylindole-1-carboxylic acid as fine white needles. The decomposition point of the acid was dependent on the rate of heating. At 2° per minute decomposition occurred at 128°, and at 8° per minute decomposition occurred at 140–141°. In each instance, skatole (m.p. 92°) and carbon dioxide were the decomposition products.

Anal.—Calcd. for $C_{10}H_9NO_2$: C, 68.56; H, 5.17; N, 7.97. Found: C, 68.82; H, 5.08; N, 7.72.

1-Formyl-3-methylindole and 3-Methyl-2-indolealdehyde.—While cooling and stirring, 42.2 Gm. (0.253 mole) of phosphorus oxychloride was added to 20.0 Gm. (0.253 mole) of N,N-dimethylformamide over a period of 30 minutes. The resulting complex was dissolved in 65 ml. of ethylene chloride and cooled to 5° before 12.8 Gm. (0.25 mole) of skatole in 100 ml. of ethylene chloride was added dropwise over a period of 1 hour. A light yellow solid separated after heating at the reflux temperature for 15 minutes. After cooling the reaction mixture, 188 Gm. (1.375 moles) of sodium acetate in 250 ml. of water was added rapidly while the reaction mixture was stirred vigorously. The organic layer was separated, decolorized with charcoal, and the solvent was evaporated. The residue was distilled at 100–120° at 0.05 mm. A saturated solution of sodium bisulfite was added to the distillate and the oily layer was removed by extraction with ethylene chloride. The aqueous layer was acidified with dilute hydrochloric acid, then extracted with ethylene chloride. Both ethylene chloride solutions were evaporated until all the solvent was removed. The second solution gave 8 Gm. (20%) of 3-methyl-2-indolealdehyde, m.p. 139–140° (8). The oily residue from the first ethylene chloride solution was distilled *in vacuo* and gave 22 Gm. (55%) of 1-formyl-3-methylindole, b.p. 98–100° at 0.03 mm., $n_D^{18.5} = 1.6138$.

The structure of 1-formyl-3-methylindole was demonstrated by the facile hydrolysis into sodium formate and skatole, by the infrared spectrum, and by elemental analysis.

Anal.—Calcd. for $C_{10}H_9NO$: C, 75.45; H, 5.70; N, 8.88. Found: C, 75.66; H, 5.83; N, 9.00.

Rearrangement of 1-Formyl-3-methylindole.—A small amount of 3-methyl-2-indolealdehyde was obtained by heating 5 Gm. of 1-formyl-3-methylindole with a trace of zinc chloride in a nitrogen-filled Carius tube at 240–270° for 2 hours. The resulting black tar-like material was distilled under high vacuum leaving 2 Gm. of tar. The distillate was treated with sodium bisulfite solution to separate the aldehyde from the amide. A 2.8-Gm. quantity of starting material was recovered and about 0.1 Gm. of impure 3-methyl-2-indolealdehyde, m.p. 130–134° was obtained.

5-(N,N-Diethylcarbamyl)-1-methyl-2-(3-methyl-2-indolylethenyl)pyridinium Iodide.—A solution of 9.1 Gm. (0.057 mole) of 3-methyl-2-indolealdehyde and 18.0 Gm. (0.057 mole) of 5-(N,N-diethylcarbamyl)-1,2-dimethylpyridinium iodide in 300 ml. methanol which contained ten drops of piperidine was refluxed for 22 hours. After chilling overnight, fine orange crystals separated. After crystallization from ethanol, 21.5 Gm. (79%) of the product, m.p. 275° dec. was obtained.

Anal.—Calcd. for $C_{22}H_{26}IN_3O$: C, 55.59; H, 5.51; N, 8.84. Found: C, 55.68; H, 5.42; N, 8.38.

1-[6-(3-Indolylethenyl)nicotinoyl]piperidine Methiodide.—In a procedure similar to that given above, 10 Gm. (0.0303 mole) of 1-(6-methylnicotinoyl)piperidine methiodide and 5.5 Gm. of 3-indolealdehyde were allowed to react. After collection and purification of the product, 10.3 Gm. (72%) of a red compound was obtained m.p. 234–236° dec.

Anal.—Calcd. for $C_{22}H_{24}IN_3O$: C, 55.86; H, 5.11; N, 8.93. Found: C, 55.90; H, 5.34; N, 8.80.

5-Ethyl-2-(3-indolylethyl)-1-methylpiperidine.—A solution of 9.35 Gm. (0.0238 mole) of 5-ethyl-2-(3-indolylethenyl)-1-methylpyridinium iodide in 250 ml. of ethanol was hydrogenated at an initial pressure of 50 p.s.i. using 2 Gm. of 5% rhodium on alumina as the catalyst. The theoretical amount of hydrogen was absorbed during 9 hours at room temperature. Removal of the catalyst and evaporation of the solvent left the hydriodide salt as a syrup. This was not purified but converted directly to the free base by the addition of sodium hydroxide. The viscous, sticky compound was partially dried by azeotropic distillation with benzene. The balance of the moisture was removed by allowing the compound to stand over phosphorus pentoxide *in vacuo* for 3 weeks. Attempts to crystallize the compound from a variety of solvents were not successful. It was necessary to dry the compound *in vacuo* after each attempt. Attempts to purify the compound as the hydrochloride salt were also unsuccessful. The salt, although readily formed, was extremely hygroscopic. The micro melting point of the compound was 135–139°.

Anal.—Calcd. for $C_{18}H_{26}N_2$: Basic N, 5.18. Found: Basic N, 4.88.

1,5-Diethyl-2-(3-indolylethyl)piperidine.—1,5-Diethyl-2-(3-indolylethenyl)pyridinium iodide, 11.25 Gm. (0.0275 mole), was hydrogenated in the same manner as described above over a period of 30 hours. The product was isolated as above but remained as a semisolid after storage over phosphorus pentoxide *in vacuo* for 1 year.

Anal.—Calcd. for $C_{19}H_{28}N_2$: Basic N, 4.94; Found: Basic N, 4.55.

5-Carbamyl-1,1-dimethyl-2-(3-indolylethyl)-piperidinium Iodide.—The hydrogenation of 10.0 Gm. (0.0247 mole) of 5-carbamyl-2-(3-indolylethenyl)-1-methylpyridinium iodide in 200 ml. of ethanol was accomplished in 3 days at 100° and at 2,500 p.s.i. pressure over 0.5 Gm. of platinum oxide as the catalyst. The semisolid product obtained after removal of the catalyst and the solvent solidified upon drying *in vacuo* over phosphorus pentoxide. This salt was dissolved in methanol containing 0.0247 mole of sodium methoxide. After heating to the reflux temperature, the solvent was removed and the residue dried. Benzene was added to dissolve the or-

ganic compound and the solution was decanted from the insoluble portion. To the benzene solution was added 3.5 Gm. (0.0248 mole) of methyl iodide and this solution refluxed for 14 hours. The product separated when the solution was allowed to cool. After crystallization from ethyl acetate, 5.5 Gm. (52%) of quaternary salt was obtained. This compound does not possess a true melting point but decomposes over a wide range.

Anal.—Calcd. for $C_{18}H_{26}IN_3O$: C, 50.92; H, 6.13; N, 9.83. Found: C, 51.06; H, 6.06; N, 9.43.

5-(N,N-Diethylcarbamyl)-1-methyl-2-(3-indolylethyl)piperidine.—The low pressure hydrogenation of 5-(N,N-diethylcarbamyl)-2-(3-indolylethenyl)-1-methylpyridinium iodide has been described (1). At low pressure this reaction was very slow, but the uptake of hydrogen was steady. The reaction at 2,500 p.s.i. pressure over platinum oxide requires only 24 hours. The resulting hydriodide was converted to the free base as described in the preceding example. This compound was identical with that previously obtained by low pressure hydrogenation (mixed m.p., 213–214°).

Anal.—Calcd. for $C_{21}H_{31}N_3O$: Basic N, 4.11. Found: Basic N, 4.08.

5-(N,N-Diethylcarbamyl)-1,1-dimethyl-2-(3-indolylethyl)piperidinium Iodide.—A benzene solution of 5-(N,N-diethylcarbamyl)-2-(3-indolylethyl)-1-methylpiperidine was allowed to react with methyl iodide in the usual fashion. The product was recrystallized from ethyl acetate under anhydrous conditions. This quaternary salt decomposed over a wide range at about 200° with the evolution of gas.

Anal.—Calcd. for $C_{22}H_{34}IN_3O$: C, 54.66; H, 7.09. Found C, 54.41; H, 7.14.

5-(N,N-Diethylcarbamyl)-1-methyl-2-(3-methyl-2-indolylethyl)piperidine Hydriodide.—A solution containing 10.0 Gm. (0.0221 mole) of 5-(N,N-diethylcarbamyl)-1-methyl-2-(3-methyl-2-indolylethenyl)pyridinium iodide in 200 ml. of ethanol was hydrogenated at 2,500 p.s.i. over 0.5 Gm. of platinum oxide at 90° for 2 days. The solid remaining after removal of the catalyst and solvent was dried *in vacuo*. Platelets were obtained after crystallization from ethyl acetate, m.p. 113–115°.

Anal.—Calcd. for $C_{22}H_{34}N_3O \cdot HI$: C, 54.66; H, 7.09. Found: C, 54.59; H, 6.97.

5-(N,N-Diethylcarbamyl)-1-methyl-2-(3-methyl-2-indolylethyl)piperidine.—The hydriodide salt described above was dissolved in methanol. An excess of sodium methoxide was added to this solution and the mixture heated at the reflux temperature. The solvent was removed by flash evaporation; the resulting semisolid was dried. The residue was extracted with benzene, the solution decolorized, and the benzene evaporated. An analytical sample was prepared by crystallization from ligroin under anhydrous conditions, mmp 158–161°.

Anal.—Calcd. for $C_{22}H_{33}N_3O$: Basic N, 3.94. Found: Basic N, 3.58.

5-(N,N-Diethylcarbamyl)-1-methyl-2-(3-methyl-2-indolylethyl)piperidine Acid Tartrate.—The free base described above (about 0.02 mole) was dissolved in 20 ml. of methanol. A methanolic solution containing 3.0 Gm. (0.02 mole) of *d*-tartaric acid was added to this solution. The volume of the solution was reduced to about one-half before 50 ml. of anhydrous ether was added to precipitate the salt. After decantation, the residue was crystallized from methanol-ethyl acetate. There was obtained 6.8 Gm. (61%) (based on the amount of pyridinium iodide originally hydrogenated) of the salt, m.p. 67–69° (sealed tube).

Anal.—Calcd. for $C_{22}H_{33}N_3O \cdot C_4H_6O_6$: C, 61.76; H, 7.77. Found: C, 61.33; H, 7.95.

4-[6-(3-Indolylethyl)-1-methylnipecotoyl]-morpholine Hydriodide.—A solution containing 10.0 Gm. (0.0212 mole) of 4-[6-(3-indolylethenyl)-nicotinoyl]morpholine methiodide in 200 ml. of ethanol was hydrogenated at 3,000 p.s.i. pressure and at 90° over 0.5 Gm. of platinum oxide for 1.5 days. After removal of the catalyst and solvent, the residue was crystallized from absolute ethanol to give a white salt, m.p. 109–110°.

Anal.—Calcd. for $C_{21}H_{29}N_3O_2 \cdot HI$: C, 52.18; H, 6.26; N, 8.89. Found: C, 52.31; H, 6.20; N, 8.76.

4-[6-(3-Indolylethyl)-1-methylnipecotoyl]-morpholine.—The hydriodide salt described above was dissolved in hot water and the solution was made faintly alkaline with sodium hydroxide. Water was removed by flash evaporation and then by azeotropic distillation with benzene. The benzene mixture was decolorized with charcoal after which the insoluble salts and the charcoal were removed by filtration. After evaporation of the benzene, the solid was crystallized from ethyl acetate to give 7.1 Gm. (95%) of white plates, m.p. 65–67°.

Anal.—Calcd. for $C_{21}H_{29}N_3O_2$: Basic N, 3.94. Found: Basic N, 4.01.

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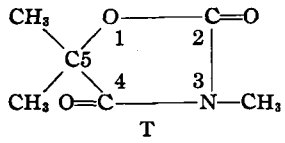
Kinetics of Base-Catalyzed Hydrolysis of Trimethadione

By JOSEPH F. GALLELLI† and H. B. KOSTENBAUDER

An infrared method is reported for determination of intact trimethadione (3,5,5-trimethyl-2,4-oxazolidinedione) in presence of products of hydrolysis. Kinetics have been determined for hydroxide ion–catalyzed hydrolysis of trimethadione and a mechanism is proposed in which there exists a rapid equilibrium between a cyclic and an acyclic structure. The rate of hydrolysis of trimethadione at pH 10 and 30° is 10^5 to 10^6 times that for acyclic compounds of similar structure.

TRIMETHADIONE (3,5,5-trimethyl-2,4-oxazolidinedione) is an anticonvulsant drug which is often administered in the form of an aqueous solution. Although it is recognized that trimethadione is rapidly decomposed in alkaline solution, the kinetics of this reaction have not been studied.

The trimethadione structure (T) can be considered as representing a lactone, a urethan (1,2,3 positions), and a cyclic amide (3,4,5 positions). Because of the multiplicity of labile



T

bonds in trimethadione, a complex mechanism of degradation is to be expected. Decomposition might occur through hydrolytic cleavage of the lactone, urethan, or amide moiety of the cyclic compound. Rekker and Nauta (1) identified products obtained on alkaline hydrolysis of trimethadione (T) as the salt of a carbamylo-α-hydroxy acid (A) and an N-methyl-α-hydroxy amide (B).

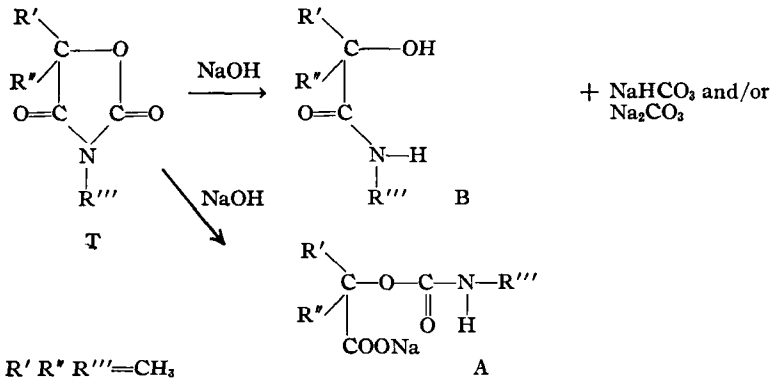
The present study was directed toward the development of an analytical procedure which would permit quantitative determination of residual quantities of intact trimethadione and toward elucidation of the kinetics and mechanism for the base-catalyzed hydrolysis of this drug.

EXPERIMENTAL

Reagents.—Trimethadione[1] U. S. P. was recrystallized twice from ethanol-water, m.p. 45–47°. The two decomposition products of trimethadione, N-methyl-α-hydroxyisobutyramide and N-methylcarbamylo-α-hydroxyisobutyric acid, were synthesized according to the method described by Rekker and Nauta (1, 2). Both compounds were recrystallized from toluene, m.p. 78–79° and m.p. 114–115°, respectively. All other chemicals were of reagent grade.

Apparatus.—A Perkin-Elmer model 137 B Infracord spectrophotometer, 0.099-mm. NaCl cell; Beckman pH meters, model H-2 and model 76; constant temperature bath ±0.01°; 1-ml. Gilmont micropipet-buret[2] with motorized syringe drive;[3] and a JKM "Stat"[4] were utilized in this study.

Kinetic Studies.—Two-gram portions of trimethadione were dissolved in distilled water in 100-ml. volumetric flasks. The solutions were brought to volume at the temperature of the bath. This solution was then transferred to a 250-ml. beaker



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[1] Marketed as Tridione by Abbott Laboratories, North Chicago, Ill.
[2] Manostat Corp., 26 N. Moore Street, New York 13, N. Y.
[3] Emil Greiner Co., New York 13, N. Y.
[4] JKM Instrument Co., Inc., Durham, Pa.

which was immersed to 90% of its length in the thermostatically controlled bath. Dipping beneath the surface of the liquid in the beaker were a glass-calomel electrode pair, a mechanical stirring device, and a 1-ml. micropipet-buret. The micropipet-buret contained a 12 *N* sodium hydroxide solution which was fed into the reaction vessel as required to maintain constant pH. Because of the high concentration of drug employed (2%) and the resulting consumption of large quantities of hydroxide ion, it was not practical to employ buffer systems to maintain constant pH. At no time did the volume of 12 *N* sodium hydroxide added exceed 0.5 ml. (0.5% of the total volume); therefore, no corrections were required to account for volume change during the reaction.

In most studies the pH in the reaction vessel was maintained by constant monitoring of pH and manual addition of sodium hydroxide solution from the micropipet-buret. For studies in the vicinity of pH 8, however, the reaction was followed for as long as 6 days and several modifications were necessitated. In these studies the reaction vessel was fitted with a rubber dam cover with a 22-gauge hypodermic needle providing an opening to avoid pressure changes. A pH stat, consisting of a Beckman model 76 expanded scale pH meter, a JKM "Stat" unit, and a motorized micropipet-buret, was employed to maintain constant pH. Maximum variation during a study was less than 0.02 pH.

At appropriate time intervals, 10-ml. portions of the aqueous alkaline reaction mixture were withdrawn and pipeted directly into 50-ml. separatory flasks. The reaction was quenched immediately by acidification to approximately pH 3 with concentrated hydrochloric acid. Trimethadione was shown to be stable at pH 3.

Determination of Intact Trimethadione.—At the end of the kinetic run each of the solutions in the separatory flasks was extracted with five 2-ml. portions of chloroform. After the addition of each portion of chloroform the separatory flask was shaken for approximately 1 minute. The chloroform extracts were filtered through a pledget of cotton into a 10-ml. volumetric flask. The cotton was rinsed with chloroform and the washings were collected in the same flask. The combined extracts were then brought to 10 ml. with chloroform and the trimethadione content of the solution was determined by measuring the absorbance at 5.49 μ, using the base-line technique (3).

An experiment was conducted in which 10-ml. portions of the aqueous alkaline solution of trimethadione were withdrawn at intervals and immediately extracted with chloroform without previous adjustment to pH 3. The chloroform extracts were then assayed as described above and the results were compared with data obtained from acid-quenching of the reaction. Both procedures gave the same result for concentration of intact trimethadione.

Determination of Decomposition Products.—The acid decomposition product, N-methyl-carbamylo-α-hydroxyisobutyric acid, was extracted from the reaction mixture with ether and was determined by titration with aqueous sodium hydroxide. A 7-Gm. quantity of anhydrous sodium sulfate was added to a 10-ml. sample of the reaction mixture from which trimethadione had been extracted. The resulting slurry was then extracted with four 10-ml. and two 5-ml. portions of ether. The combined ether extracts were evaporated and the residue was titrated with standard sodium hydroxide, approximately 0.01 *N* or 0.1 *N*.

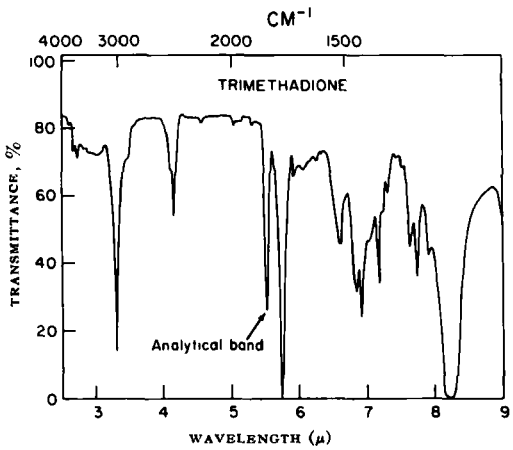


Fig. 1.—Infrared absorption spectrum for trimethadione in chloroform.

Direct determination of the amide decomposition product, N-methyl-α-hydroxyisobutyramide, in the reaction mixture was not practical; however, the reaction scheme indicates that formation of 1 mole of the amide must be accompanied by liberation of 1 mole of carbonate. Determination of carbonate in the reaction mixture, therefore, provided an indirect means of determining the quantity of amide formed.

The 10-ml. samples of reaction mixture to be used for carbonate determination were immediately acidified with 0.5 ml. of a sodium biphosphate solution (70 mg./ml.) to quench the reaction. The samples were then placed in a closed system and acidified with 1 ml. of concentrated sulfuric acid. The carbon dioxide evolved was flushed from the solution by bubbling nitrogen through the system for 1 hour and passing the effluent gases through two traps containing 30 ml. of standard barium hydroxide approximately 0.05 *N* in the first trap and 0.02 *N* in the second trap. The precipitate of barium carbonate was allowed to settle and a clear 10-ml. aliquot of the remaining barium hydroxide solution in each trap was titrated with 0.05 *N* hydrochloric acid to determine the equivalents of barium hydroxide precipitated by the carbon dioxide.

NMR Data.—A nuclear magnetic resonance spectrum[5] was obtained to confirm the structure of one of the products of decomposition, the N-methyl-α-hydroxyisobutyramide. The sample was run as a saturated solution in $CDCl_3$.

RESULTS AND DISCUSSION

Specificity of Assay for Intact Trimethadione.—Previously reported assay methods for trimethadione consist of determination of total nitrogen (4), determination of alkali consumed during hydrolysis in strong base (5), and measurement of carbon

[5] Furnished through the courtesy of Varian Associates, Palo Alto, Calif.

dioxide evolved on acidification after alkaline hydrolysis (6). The assays are unsatisfactory for distinguishing intact drug from decomposition products and for the last two assays the results obtained, even with pure trimethadione, are highly dependent on the conditions under which the assay is conducted.

Trimethadione is transparent in the visible and ultraviolet regions of the spectrum but exhibits a distinctive absorption band in the infrared at 5.49 μ. This absorption is similar to that exhibited by lactones (7); this band disappears upon destruction of the cyclic structure.

None of the possible decomposition products of trimethadione show absorption at 5.49 μ. Therefore, determination of absorbance of a chloroform extract of the aqueous reaction medium provides an assay specific for intact drug. Figure 1 shows the infrared spectrum of trimethadione and Fig. 2 shows the standard curve of absorbance *versus* concentration.

Specificity of Assays for Decomposition Products.—The product N-methyl-carbamylo-α-hydroxyisobutyric acid was determined by ether extraction of acidified aqueous solution, followed by evaporation and titration of residue with standard alkali. Presumably a product of further hydrolysis, α-hydroxyisobutyric acid, might also respond to such a procedure. It has been reported (1) that the sodium salt of carbamylo-α-hydroxy acid is not sensitive to alkali even at 100°; however, prolonged exposure to excess alkali did result in further hydrolysis. Below pH 10.8, however, it was found that there was a loss of less than 1% per hour. It would appear that for the duration and conditions of the present studies the carbamylo-α-hydroxyisobutyric acid can properly be considered as a final product.

The possibility of cleavage at the 2,3 position yielding another acid product of equivalent weight identical to N-methyl-carbamylo-α-hydroxyisobutyric acid was eliminated on examination of NMR

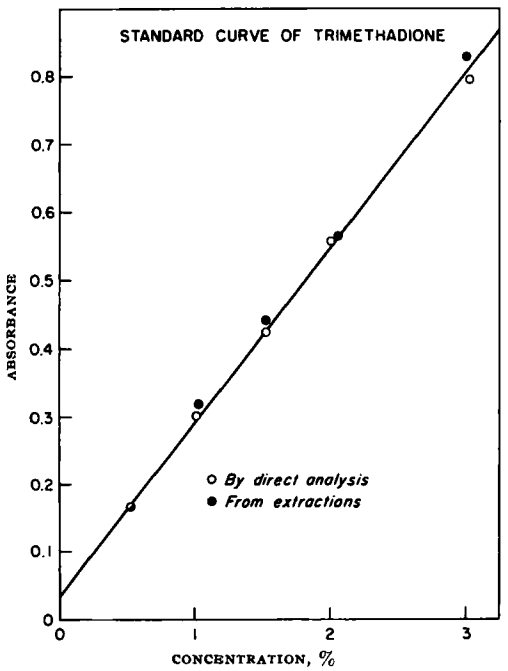


Fig. 2.—Standard curve for infrared determinations of trimethadione in chloroform. The open circles represent trimethadione added to chloroform; the solid circles represent chloroform extractions of aqueous solutions of trimethadione.

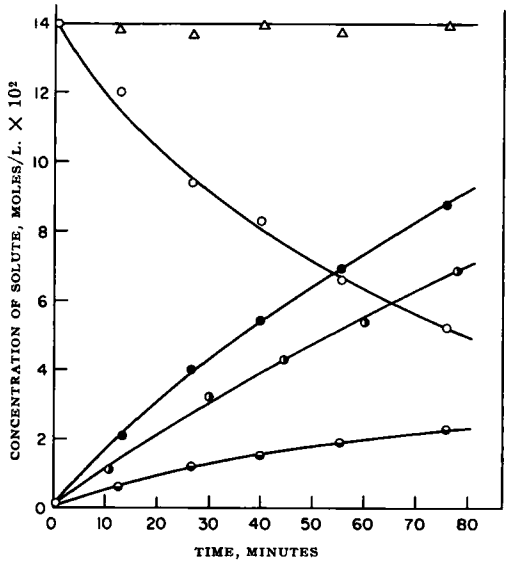


Fig. 3.—Verification of stoichiometry of trimethadione hydrolysis at pH 10 and 30°. Key: ○, trimethadione; ◑, N-methyl-α-hydroxyisobutyramide; ◑, N-methyl-carbamylo-α-hydroxyisobutyric acid; ●, ◑ plus ⊖; △, total.

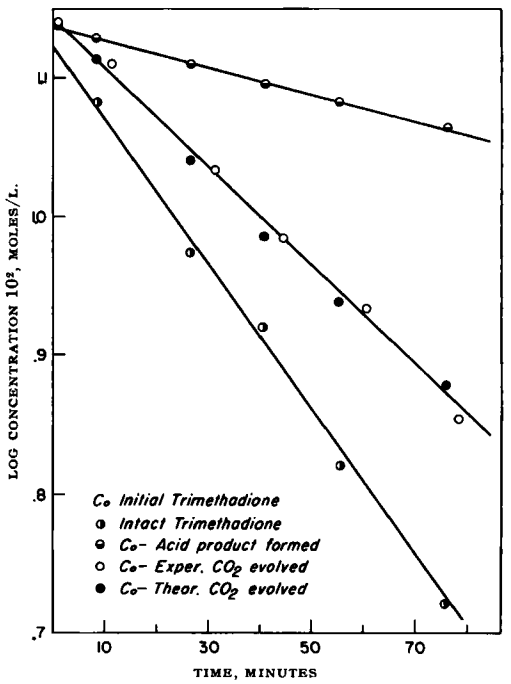
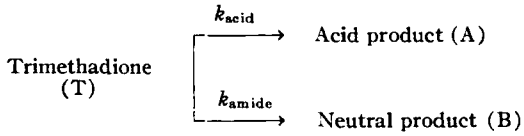


Fig. 4.—First-order disappearance of trimethadione and appearance of products.

spectra obtained for the acid product. The alternate structure for the acidic component would require the resonance of the N-methyl to be single, since there is no adjacent proton; the resonance is, in fact, doubled indicating that the N atom carries a proton as required for the N-methyl-carbamylo-α-hydroxyisobutyric acid structure. Additional resonance assignments and the integrated intensities confirm the above structural assignment.

Adherence to stoichiometry during the course of the reaction, as illustrated in Fig. 3, supports the acceptability of the determination of liberated carbon dioxide as an indirect means of determining the quantity of N-methyl-α-hydroxyisobutyramide produced.

Order of Reaction with Respect to Trimethadione. —Figure 4 illustrates that overall disappearance of trimethadione is first order with respect to intact drug. The nature of Fig. 3 suggests that at constant hydroxide ion concentration the decomposition occurs by formation of an acid product and an amide *via* two parallel first-order reactions. The proposed reaction is



The symbols T, A, B represent trimethadione, N-methyl-carbamylo-α-hydroxyisobutyric acid, and N-methyl-α-hydroxyisobutyramide, respectively, and k_{acid} and k_{amide} represent the corresponding rate constants. The rate equation at constant hydroxide ion concentration is

$$\frac{-dT}{dt} = \frac{dA}{dt} + \frac{dB}{dt} \qquad \text{(Eq. 1)}$$

or $$-\frac{dT}{dt} = kT = k_{acid}T + k_{amide}T \qquad \text{(Eq. 2)}$$

where $$k = k_{acid} + k_{amide} \qquad \text{(Eq. 3)}$$

and $$ln\ (T_0/T) = kt \qquad \text{(Eq. 4)}$$

or $$T = T_0e^{-kt} \qquad \text{(Eq. 5)}$$

where T_0 is the initial concentration of trimethadione and T is the concentration at any time t. The rate of formation of the acidic product can be expressed as

$$\frac{dA}{dt} = k_{acid}T \qquad \text{(Eq. 6)}$$

$$= k_{acid}T_0e^{-kt} \qquad \text{(Eq. 7)}$$

and by integration

$$A = -\frac{k_{acid}T_0e^{-kt}}{k} + \text{constant} \qquad \text{(Eq. 8)}$$

$$A = A_0 + \frac{k_{acid}}{k}T_0(1 - e^{-kt}) \qquad \text{(Eq. 9)}$$

where A_0 is the initial concentration of the acid product and A is the concentration at any time t. If $A_0 = 0$ (Eq. 10), then

$$A = \frac{k_{acid}}{k}T_0(1 - e^{-kt}) \qquad \text{(Eq. 11)}$$

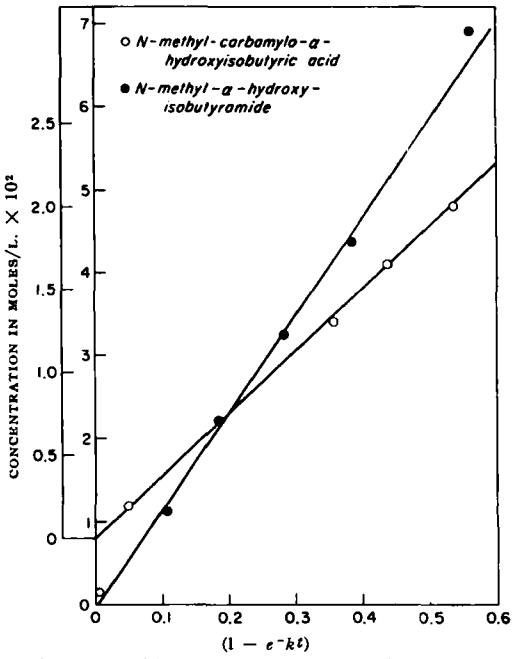


Fig. 5.—Evidence for parallel pseudo-first order reactions for hydrolysis of trimethadione to N-methyl-carbamylo-α-hydroxyisobutyric acid and N-methyl-α-hydroxyisobutyramide at pH 10.0 and 30°.

A similar expression derived for the neutral product is

$$B = \frac{k_{amide}}{k}T_0(1 - e^{-kt}) \qquad \text{(Eq. 12)}$$

Equations 11 and 12 indicate that for the base catalyzed hydrolysis of trimethadione, a plot of the concentration of either A or B against $(1 - e^{-kt})$ should yield a straight line. At zero time the curve should pass through the origin and at infinite time the function should have a value of unity.

The value for k, the overall first-order rate constant, was obtained by plotting logarithm of the concentration of residual trimethadione *versus* time at various hydroxide ion concentrations. It was then possible to check the relationship expressed in Eq. 11 using the concentrations of acidic product formed at various hydroxide ion concentrations. A plot of the acidic product formed in the degradation of trimethadione at pH 10.0 and 30° *versus* $(1 - e^{-kt})$ yielded a straight line through the origin as predicted by Eq. 11 (See Fig. 5). Also shown in Fig. 5 is a linear plot of the neutral or amide product formed at pH 10.0 and 30° *versus* $(1 - e^{-kt})$. The value of k_{acid}, the rate constant for the formation of acidic material, was calculated from the slope by Eq. 11.

$$\text{Slope} = \frac{k_{acid}}{k}T_0 \qquad \text{(Eq. 13)}$$

The value of k_{amide}, the rate constant for the formation of neutral material at pH 10.0 and 30°, was also obtained in the same manner. Values for k_{amide} at other pH values were obtained by evaluat-

ing k_{acid} in the above manner at each pH value and then subtracting k_{acid} from k according to Eq. 3. The data are found in Table I.

Hydroxide Ion Dependency.—The influence of hydroxide ion on the rate constant for trimethadione decomposition is illustrated in Fig. 6. Also shown in Fig. 6 is the influence of hydroxide ion on the rate constants for appearance of the decomposition products, N-methyl-carbamylo-α-hydroxyisobutyric acid and N-methyl-α-hydroxyisobutyramide. All plots show a hydroxide ion dependency of an order that is non-integral but greater than one.

Temperature Dependency.—Rate constants for the overall disappearance of trimethadione were obtained over the temperature range of 30 to 60° at a pH of 9.25. The data are presented as an Arrhenius plot in Fig. 7, and the apparent heat of activation for the process was determined as approximately 26.0 kilocalories per mole.

Mechanism.—Over the pH range studied, the pseudo-first order rate coefficient for the disappearance of trimethadione and the rate coefficients for the parallel pseudo-first order appearance of decomposition products, N-methyl-α-hydroxyisobutyramide and N-methyl-carbamylo-α-hydroxyisobutyric acid, all show hydroxide ion dependencies which are non-integral but of an order greater than one. The amide is the principal product below about pH 8.75, with the acid product becoming increasingly important above pH 9.50.

In postulating a mechanism to satisfy these requirements, the data illustrated in Fig. 8, showing hydroxide ion uptake during the reaction, become extremely useful. Figure 8 indicates an initial uptake of hydroxide ion greater than the equivalent quantity of trimethadione disappearing, followed by a region in which the hydroxide uptake is proportional to the trimethadione disappearing but is less than equivalent to the trimethadione disappearing. This observation suggests a relatively rapid reaction of hydroxide ion with trimethadione to produce a species which is in equilibrium with the trimethadione. On the basis of these observations the mechanism proposed is

TABLE I.—EXPERIMENTAL RATE CONSTANTS FOR THE BASE-CATALYZED HYDROLYSIS OF TRIMETHADIONE AT 30° C.

pH	k, Hr.$^{-1}$	k_{acid}, Hr.$^{-1}$	k_{amide}, Hr.$^{-1}$
10.75	7.314	2.710	4.604
10.50	2.696	0.873	1.823
10.25	1.325	0.359	0.966
10.00	0.676	0.145	0.531
9.75	0.292	0.047	0.245
9.50	0.119	0.019	0.100
9.25	0.0684	...	...
9.00	0.0284	...	...
8.75	0.0141	...	...
8.00	0.00251	...	...

Development of a rate expression can be considerably simplified if it can be assumed that the cyclic structure I is in rapid equilibrium with the acyclic structure III. An equilibrium quotient may then be written as

$$K = \frac{\text{III}}{(\text{I})\,(\text{OH}^-)} \qquad \text{(Eq. 14)}$$

The fraction of total trimethadione present in the cyclic form is

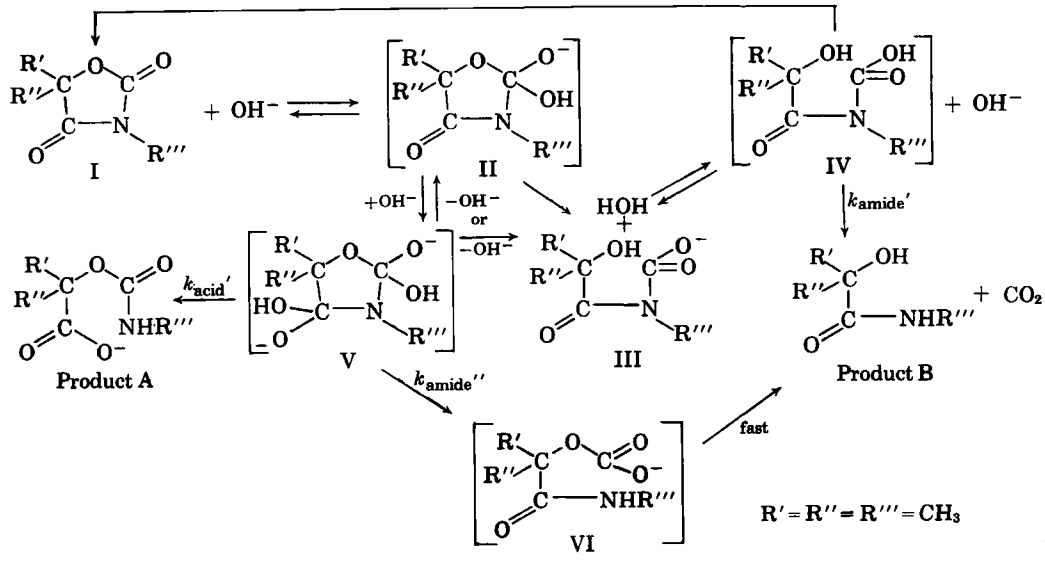
$$\frac{1}{1 + K\,(\text{OH}^-)} \qquad \text{(Eq. 15)}$$

and the fraction of total trimethadione in the acyclic form III is

$$\frac{K\,(\text{OH}^-)}{1 + K\,(\text{OH}^-)} \qquad \text{(Eq. 16)}$$

The existence of this equilibrium could not be verified directly, since at a pH where a significant fraction of trimethadione would theoretically exist in the acyclic structure, hydrolysis is extremely rapid. Structure IV apparently is extremely unstable, for in no case was it possible to isolate this compound after acidification of the reaction mixture.

In the following development, T represents total trimethadione in solution (essentially I + III), K is the equilibrium quotient as defined above, and



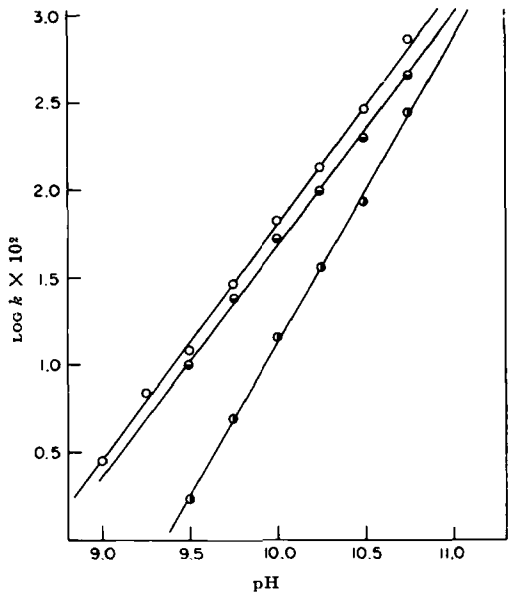


Fig. 6.—Influence of hydroxide ion on the pseudo-first order rate constant for hydrolysis of trimethadione at 30°. Also shown is the effect of hydroxide ion on the rate constants for appearance of products. Key: O, trimethadione; ◒, N-methyl-α-hydroxyisobutyramide; ◑, N-methyl-carbamylo-α-hydroxyisobutyric acid.

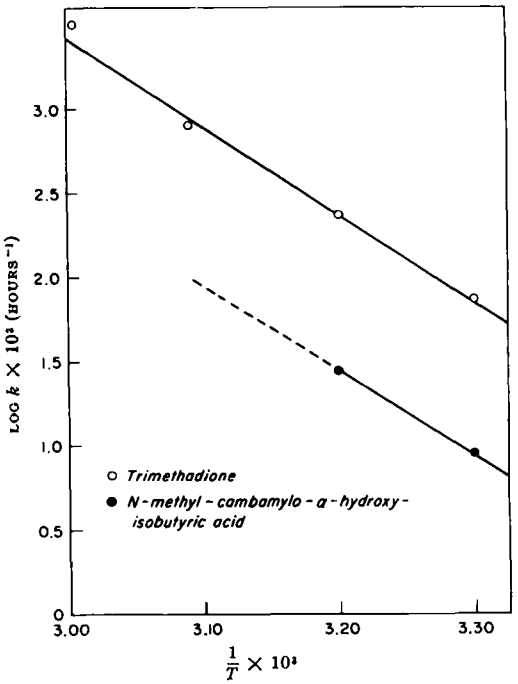


Fig. 7.—Arrhenius-type plots showing the temperature dependency for hydrolysis of trimethadione and appearance of N-methyl-carbamylo-α-hydroxyisobutyric acid at pH 9.25.

$k_{amide'}$, $k_{amide''}$, and $k_{acid'}$ are rate coefficients for formation of decomposition products. The overall rate of disappearance of trimethadione can be represented as

$$\text{Rate} = (\text{III})\,k_{amide'} + (\text{I})\,(OH^-)^2\,k_{amide''} + (\text{I})\,(OH^-)^2\,k_{acid'} \quad \text{(Eq. 17)}$$

When Eqs. 15 and 16 are substituted into Eq. 17, then

$$\text{Rate} = \frac{(T)\,K\,(OH^-)\,k_{amide'}}{1 + K\,(OH^-)} + \frac{(T)\,(OH^-)^2\,k_{amide''}}{1 + K\,(OH^-)} + \frac{(T)\,(OH^-)^2\,k_{acid'}}{1 + K\,(OH^-)} \quad \text{(Eq. 18)}$$

or

$$\frac{\text{Rate}}{T} = \frac{K\,(OH^-)\,k_{amide'}}{1 + K\,(OH^-)} + \frac{(OH^-)^2\,k_{amide''}}{1 + K(OH^-)} + \frac{(OH^-)^2\,k_{acid'}}{1 + K\,(OH^-)} \quad \text{(Eq. 19)}$$

The constants in Eq. 19 are evaluated in the following manner. At any constant hydroxide concentration, the observed pseudo-first order rate coefficients listed in Table I may be expressed as

$$k_{amide} = \frac{K\,(OH^-)\,k_{amide'}}{1 + K\,(OH^-)} + \frac{(OH^-)^2\,k_{amide''}}{1 + K\,(OH^-)} \quad \text{(Eq. 20)}$$

$$k_{acid} = \frac{(OH^-)^2\,k_{acid'}}{1 + K\,(OH^-)} \quad \text{(Eq. 21)}$$

$$k = k_{acid} + k_{amide} \quad \text{(Eq. 22)}$$

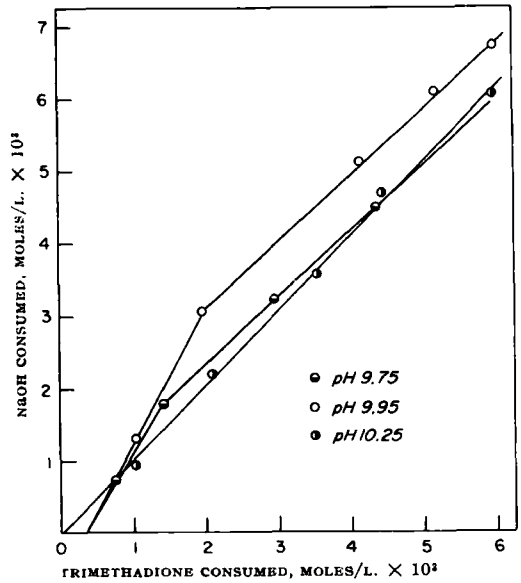


Fig. 8.—Plots showing uptake of hydroxide ion *versus* quantity of trimethadione degrading during hydrolysis reaction.

Using Eq. 21 and substituting observed values for k_{acid} at several hydroxide ion concentrations, it is possible to solve for the two unknowns, $k_{acid'}$ and K. These constants were determined from data obtained in the pH range 9.50 to 10.75. Using Eq. 20 and substituting the known value for K, and selecting values for k_{amide} at several hydroxide ion concentrations from Table I, simultaneous equa-

tions may be employed to determine $k_{amide'}$ and $k_{amide''}$. The coefficients thus calculated are

$$k_{acid'} = 0.965 \times 10^{7}\ \text{hr.}^{-1}$$
$$k_{amide'} = 0.074 \times 10^{1}\ \text{hr.}^{-1}$$
$$k_{amide''} = 1.491 \times 10^{7}\ \text{hr.}^{-1}$$
$$K = 2.564 \times 10^{3}$$

Using the above coefficients in the overall rate equation (Eq. 19) the theoretical rate of disappearance of trimethadione was calculated for the pH range of 7.5 to 11.0. The calculated rate appears as a solid line in Fig. 9. The rate expression predicts that the overall rate for disappearance of trimethadione should be first order with respect to hydroxide ion below pH 8.75 and above pH 11.0, and that the order is non-integral between pH 8.75 and 11.0. The experimentally determined rates are in good agreement with the theoretical and the rate does become first order with respect to hydroxide ion below pH 8.75. It was not feasible to study the reaction above pH 11.0 to examine for first-order hydroxide ion dependency because of the extremely rapid reaction under these conditions.

Comparison of Hydrolysis Rates for Cyclic and Acyclic Compounds.—Trimethadione is much more sensitive to alkaline hydrolysis than are acyclic compounds of similar structure. At pH 10 and 30° trimethadione is hydrolyzed at a rate approximately 10^{6} times that of ethyl-N-methylcarbamate (8), and approximately 10^{5} times the rate at which ethyl carbamate (urethan) (8) is hydrolyzed under similar conditions.

Rekker and Nauta (1) demonstrated that, in the presence of alkali, carbamylo-α-hydroxy esters are not hydrolyzed directly but are first converted to a trimethadione-type cyclic intermediate which then hydrolyzes to produce as products an N-substituted-α-hydroxy amide and the salt of a carbamylo-α-hydroxy acid. (See reaction scheme below.)

The extreme sensitivity of the trimethadione structure to alkaline hydrolysis (when compared with acyclic structures) suggests that the trimethadione structure might be of interest as an enzyme model or as an intermediate in the decomposition or metabolism of related drugs.

Pharmaceutical Significance.—From the theoretical rate equation proposed, half-life values for hydrolysis of trimethadione were calculated as a function of pH and are listed in Table II. It should be noted that these values are for solutions in which constant hydroxide ion concentration is maintained throughout the course of the reaction. Since trimethadione is usually employed in high concentration (5%) and since hydroxide ion is consumed in the hydrolysis reaction, the reaction presumably is self-limiting in unbuffered or weakly buffered solutions.

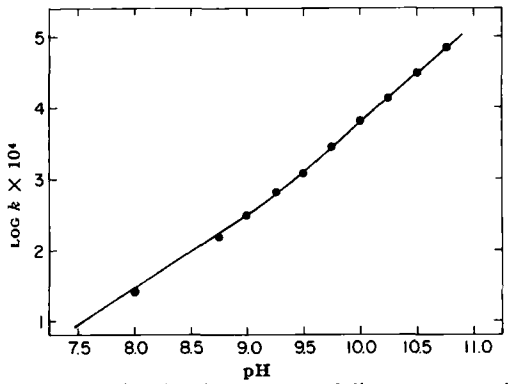


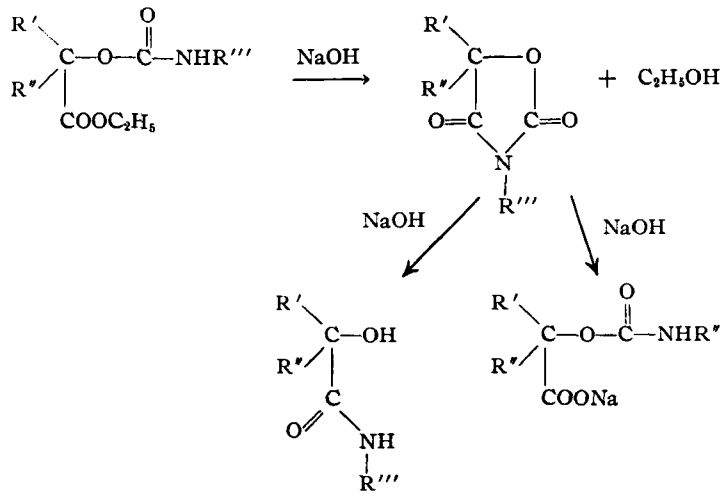
Fig. 9.—Plot showing the rate of disappearance of trimethadione as a function of hydroxide ion concentration over the pH range 8.0 to 11.0. The theoretical curve as calculated from the rate equation is shown as a solid line. The circles are experimental points determined at 30°.

TABLE II.—HALF-LIFE PERIODS, $t^1/_2$, FOR THE DEGRADATION OF TRIMETHADIONE AT CONSTANT pH AND 30° C. CALCULATED FROM THE THEORETICAL RATE EQUATION

pH	Half-life, hr.
7.0	2472
8.0	247
9.0	25.3
10.0	1.11
11.0	0.047

SUMMARY

1. An infrared method is reported for determination of trimethadione in the presence of products of its hydrolytic decomposition.



2. The kinetics of the base catalyzed hydrolysis of trimethadione (3,5,5-trimethyl-2,4-oxazolidinedione) have been determined for the pH range 8.0 to 10.75 at 30°. Pseudo–first order rate coefficients are reported for the disappearance of trimethadione and for the parallel pseudo–first order appearance of the decomposition products N-methyl-carbamylo-α-hydroxyisobutyric acid and N-methyl-α-hydroxyisobutyramide. The amide is the principal product below pH 8.75, with the acid becoming an increasingly important product above pH 9.50.

3. The overall rate of disappearance of trimethadione in aqueous solution, at constant hydroxide ion concentration, was found to be in agreement with a rate equation of the form

$$\frac{\text{Rate}}{T} = \frac{K(OH^-)\,k_{amide'}}{1 + K(OH^-)} + \frac{(OH^-)^2\,k_{amide''}}{1 + K(OH^-)} + \frac{(OH^-)^2\,k_{acid'}}{1 + K(OH^-)}$$

4. A mechanism was proposed in which there exists a rapidly reversible equilibrium between the cyclic trimethadione and an acyclic structure.

5. Trimethadione is much more sensitive to alkaline hydrolysis than acyclic compounds of similar structure. Trimethadione hydrolyzes approximately 1,000,000 times as fast as ethyl-N-methylcarbamate and approximately 100,000 times as fast as ethyl carbamate (urethan) at pH 10 and 30°.

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Determination and Identification of *p*-Hydroxyamphetamine as the O,N-Diacetyl Derivative

By LLEWELLYN H. WELSH and A. FRANCIS SUMMA

Therapeutic solutions of hydroxyamphetamine hydrobromide, such as the ophthalmic solution U.S.P. and nasal solution, N.N.R. (N.N.D.), may be assayed gravimetrically by treating with acetic anhydride in the presence of bicarbonate and quantitatively isolating the O,N-diacetylhydroxyamphetamine thus formed. The properties of the readily crystallizable derivative serve to identify the parent substance.

TESTS AND STANDARDS for New and Nonofficial Remedies" (1) includes monographs for a 1% nasal solution and a 1% ophthalmic solution of *p*-hydroxyamphetamine hydrobromide. The assay specified for the former solution involves liberation of hydroxyamphetamine base with potassium carbonate, extraction of the base into ether, addition of excess standard acid to the extract, and back-titration after evaporation of the ether. The assay specified for the latter solution is based on measurement of its absorbance at 225 mμ.

Vincent, Krupski, and Fischer (2) have reported an alkalimetric method in which the solution containing a salt of hydroxyamphetamine is passed through a column of Amberlite IR-45. The base so formed is titrated after being eluted with ethanol. Varga and Vastagh (3) have developed a bromometric assay applicable to therapeutic solutions of hydroxyamphetamine.

The alkalimetric methods and the procedure of Varga and Vastagh are relatively nonspecific. In regulatory work it would be necessary to supplement them with experimental data providing some assurance that consumption of reagent is due only to the substance to be quantitated. The N.N.R. (1) alkalimetric method is, in addition, somewhat tedious. Even with the salting-out effect produced by the specified high concentration of potassium carbonate (15 Gm. for a 20-ml. sample), seven extractions are required, and there are other difficulties related to the low specific gravity and high vapor pressure of ether. The N.N.R. spectrophotometric method, although convenient, is applicable only in the laboratory of the manufacturer since it requires employing as a blank the menstruum used in preparing the solution.

In the course of investigating alternative

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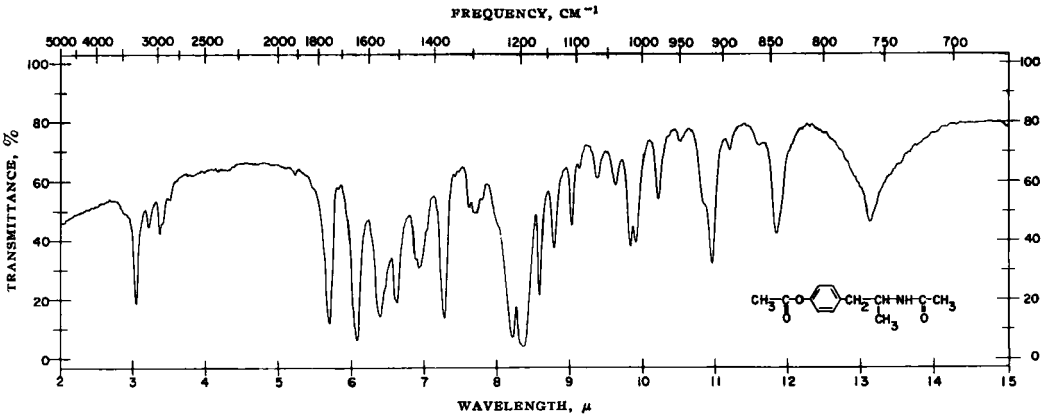

Fig. 1.—Infrared absorption spectrum of crystalline O,N-diacetyl-*p*-hydroxyamphetamine (1 mg.) in 0.5 in. (200 mg.) KBr disk. Recorded with a Perkin-Elmer model 21 spectrophotometer.

methods of assay acceptable for regulatory use, application was made of the general procedure for acetylation which has proved useful in the analysis of other sympathomimetic amines (4–6). Under the conditions of the procedure (dilute solution of the drug in *ca.* 10% aqueous bicarbonate), *p*-hydroxyamphetamine reacts rapidly and quantitatively with acetic anhydride to form the O,N-diacetyl derivative which may be extracted easily into chloroform and weighed after removal of solvent. This is the basis of the assay presently proposed. The method is manipulatively less difficult than the N.N.R. alkalimetric assay, requires one-half as much sample (10 ml.), and is applicable to individual units of the size most commonly marketed (15 ml.).[1] Furthermore, it provides a readily crystallizable derivative, the properties of which serve to identify the drug and give an indication of its purity.[2]

EXPERIMENTAL

Assay.—Pipet 10 ml. of hydroxyamphetamine hydrobromide solution (1%) into a 125-ml. separator. Extract with 15 ml. of chloroform; discard the extract. Rinse the stopper and mouth of the separator with a few drops of water and allow the rinsings to combine with the contents. Add 1.05 Gm. of sodium bicarbonate, preventing it from contacting the mouth of the separator, and swirl until most of the bicarbonate has dissolved. By means of a 1-ml. syringe, rapidly inject 0.50 ml. of acetic anhydride into the contents of the separator, stopper the vessel immediately, and shake it vigorously until the evolution of carbon dioxide has ceased, releasing the pressure as necessary through the stopcock. Allow to stand for 5 minutes and extract the solution with five 10-ml. portions of chloroform; filter each extract through a pledget of cotton (previously washed with chloroform) into a beaker.

Evaporate the combined extracts on a steam bath in a current of air to about 3 ml., completely trans-

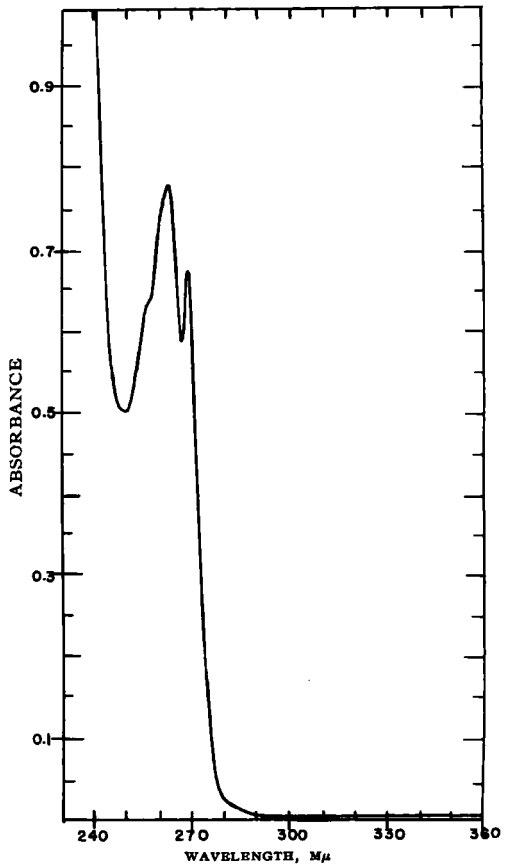

Fig. 2.—Ultraviolet absorption spectrum of O,N-diacetyl-*p*-hydroxyamphetamine in 95% ethanolic solution (400 mg./L., 1-cm. cell). Recorded with a model DK-2 Beckman spectrophotometer: ϵ_{265} = 368, ϵ_{271} = 315.

[1] The assay is now part of the monograph for hydroxyamphetamine hydrobromide ophthalmic solution, recently recognized by the U.S.P. (7).

[2] Identification of the product obtained in the assay does not establish unequivocally the identity of the solute since the same substance would be isolated if the solution originally contained *p*-hydroxyamphetamine, its diacetyl derivative, either of the two possible monoacetyl derivatives, or any combination of the four substances. However, the possibility would appear remote that any one of the relatively inaccessible acetylation products would find its way into a commercial preparation either by accident or design.

fer the concentrate by means of small portions of chloroform to a tared 50-ml. beaker, and continue the evaporation until the solvent is completely removed. Heat the residue of diacetyl derivative at 80° for 90 minutes, cool in a desiccator and weigh. (Weight of the residue × 0.9866 = weight of *p*-hydroxyamphetamine hydrobromide.)

O,N - Diacetyl - *p* - hydroxyamphetamine.—The white crystalline substance (m.p. 97.5 to 98.5°) obtained in the assay was recrystallized from carbon tetrachloride (6 ml./Gm.; 95% recovery). Twice-recrystallized material melted at 98 to 98.5° and exhibited the following optical crystallographic properties:[3] (*a*) *habit*–needles, and rods, some in bundles broader at one end than at the other; (*b*) *refractive indices*–α = 1.538, β = 1.548 (common), γ = 1.604 (all ± 0.003); (*c*) *extinction*–parallel and inclined; (*d*) *optic sign*–positive; (*e*) *elongation*–positive and negative; (*f*) 2*V*–moderately small.

The infrared and ultraviolet spectra are shown in Figs. 1 and 2, respectively.

Anal.–Calcd. for $C_{13}H_{17}NO_3$: C, 66.36; H, 7.28; N, 5.95. Found:[4] C, 66.30; H, 7.47; N, 6.03.

RESULTS AND DISCUSSION

Five assays of a 1% standard solution of hydroxyamphetamine hydrobromide[5] gave recoveries in the range of 100.2 to 100.6% (av. 100.5%). Duplicate assays of a solution prepared to contain 1% of the drug, boric acid (2%), and thimerosal (1:50,000) yielded recoveries of 100.6 and 101.0%, whereas five assays of a commercial ophthalmic solution of the same declared composition afforded results corresponding to 101.1 to 101.4% (av. 101.2%) of the declared amount of active ingredient (m.p. of isolated derivative 97.5 to 98.5°).

Coincidence of the infrared spectrum of the derivative with that of authentic diacetyl-*p*-hydroxyamphetamine is, *per se*, sufficient to identify the parent substance within the limitations discussed.[2] If infrared spectrophotometric equipment is not available, the base (8–10) may be extracted from the solution and identified by the classical methods of qualitative organic analysis. In addition to the hydrobromide (10, 11), the following easily prepared derivatives of *p*-hydroxyamphetamine have been reported in the literature: hydrochloride (9, 12–14), hydriodide (8), 2,4-dinitrobenzoic acid salt, and N-benzoyl derivative (15). Color reactions of *p*-hydroxyamphetamine and its behavior with alkaloidal precipitants have been described by Haley (16).

[3] Determined by Arnold E. Schulze, Division of Microbiology, Food and Drug Administration, U. S. Department of Health, Education, and Welfare.

[4] Microanalyses by Harold G. McCann, National Institutes of Health, U. S. Department of Health, Education, and Welfare.

[5] We are indebted to Smith Kline and French Laboratories, Philadelphia, Pa., for a generous supply of U.S.P. hydroxyamphetamine hydrobromide.

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ERRATUM

In the paper titled "Colorimetric Assay of Nystatin" (1), the ordinate markings for Figs. 1 and 2 were incorrect and are reproduced correctly here

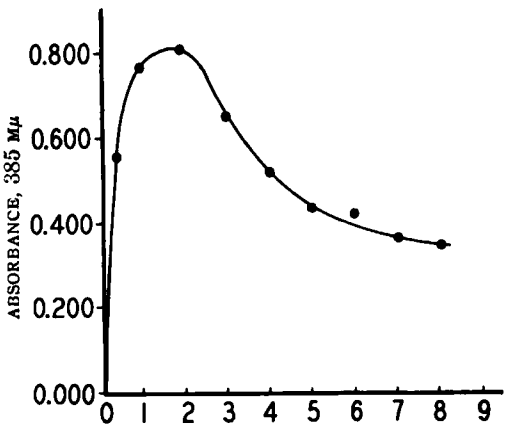

Fig. 1.—Color development of basic hydrolysis of nystatin (5080 units in 5 ml. aliquot).

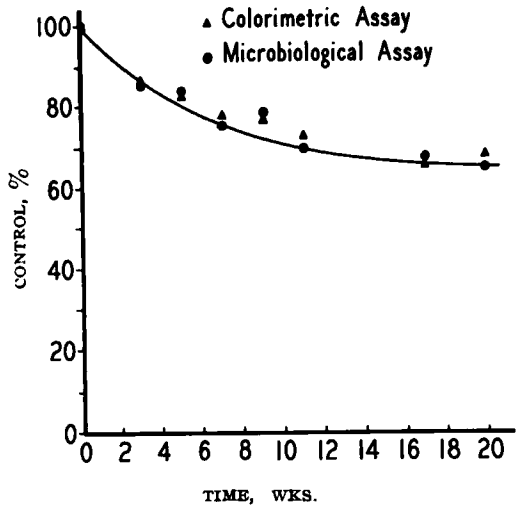

Fig. 2—Degradation of nystatin at 50° C.

(1) Chang, J. C., Honig, A. B., Warren, A. T., and Levine, S., THIS JOURNAL **52**, 536(1963).

fer the concentrate by means of small portions of chloroform to a tared 50-ml. beaker, and continue the evaporation until the solvent is completely removed. Heat the residue of diacetyl derivative at 80° for 90 minutes, cool in a desiccator and weigh. (Weight of the residue × 0.9866 = weight of *p*-hydroxyamphetamine hydrobromide.)

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RESULTS AND DISCUSSION

Five assays of a 1% standard solution of hydroxyamphetamine hydrobromide[5] gave recoveries in the range of 100.2 to 100.6% (av. 100.5%). Duplicate assays of a solution prepared to contain 1% of the drug, boric acid (2%), and thimerosal (1:50,000) yielded recoveries of 100.6 and 101.0%, whereas five assays of a commercial ophthalmic solution of the same declared composition afforded results corresponding to 101.1 to 101.4% (av. 101.2%) of the declared amount of active ingredient (m.p. of isolated derivative 97.5 to 98.5°).

Coincidence of the infrared spectrum of the derivative with that of authentic diacetyl-*p*-hydroxyamphetamine is, *per se*, sufficient to identify the parent substance within the limitations discussed.[2] If infrared spectrophotometric equipment is not available, the base (8–10) may be extracted from the solution and identified by the classical methods of qualitative organic analysis. In addition to the hydrobromide (10, 11), the following easily prepared derivatives of *p*-hydroxyamphetamine have been reported in the literature: hydrochloride (9, 12–14), hydriodide (8), 2,4-dinitrobenzoic acid salt, and N-benzoyl derivative (15). Color reactions of *p*-hydroxyamphetamine and its behavior with alkaloidal precipitants have been described by Haley (16).

[3] Determined by Arnold E. Schulze, Division of Microbiology, Food and Drug Administration, U. S. Department of Health, Education, and Welfare.

[4] Microanalyses by Harold G. McCann, National Institutes of Health, U. S. Department of Health, Education, and Welfare.

[5] We are indebted to Smith Kline and French Laboratories, Philadelphia, Pa., for a generous supply of U.S.P. hydroxyamphetamine hydrobromide.

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ERRATUM

In the paper titled "Colorimetric Assay of Nystatin" (1), the ordinate markings for Figs. 1 and 2 were incorrect and are reproduced correctly here

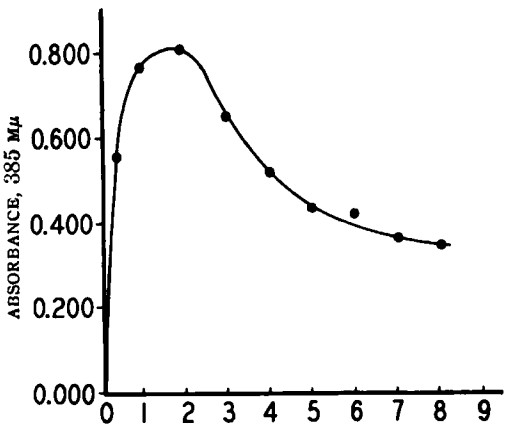

Fig. 1.—Color development of basic hydrolysis of nystatin (5080 units in 5 ml. aliquot).

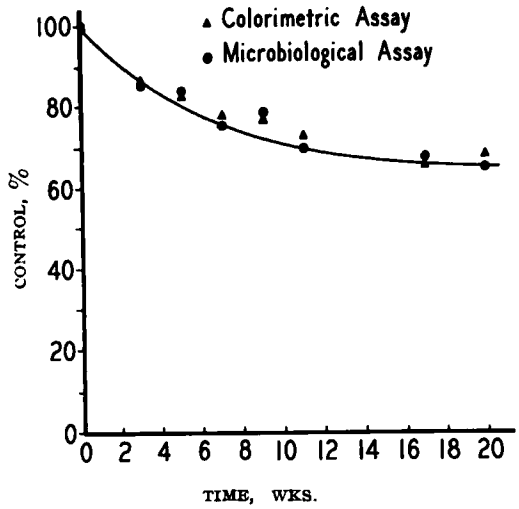

Fig. 2—Degradation of nystatin at 50° C.

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Influence of Organic Groups on the Polarographic Stability of Mercury Compounds

By SISTER M. LAWRENCE O'DONNELL, ANSELMA SCHWARZKOPF, and CORNELIUS W. KREKE

Polarograms of 26 organic mercurials with different R structures were made. The half-wave potentials of these compounds correlate qualitatively with the polarizability of the R structure and the polarities of the mercury-chloride bond in the first wave and the carbon-mercury bond in the second. The polarographic reductive stability of the ring molecules below that of the chain compounds agrees with the greater electronegativity of the rings. Compounds which have found clinical usefulness as diuretics have similar polarographic properties. It is suggested that the electron density of the mercury may be the basis of the biological selectivity of these organic compounds.

IN SPITE OF their great affinity for sulfhydryl groups, organic mercurials show considerable differences in binding with protein. While these differences may be due in some instances to the configurations of the mercurials or to affinities for buffer ions or secondary groups of the protein surface, it seems also that their individual affinities for the sulfhydryl group itself must be a factor.

As an instance of this latter phenomenon it was found that phenylmercuric hydroxide (PMOH) differed from *p*-chloro-mercuribenzoate (PCMB) in its binding on egg albumin (1). Although both have similar configurations and both are known to combine readily with sulfhydryl, PMOH reacted with one SH under the same conditions that PCMB reacted with two. Moreover, under the same conditions, PMOH showed considerable non-sulfhydryl binding while PCMB possessed no affinity for secondary sites.

In this study the half-wave potentials of a series of organic mercury compounds of the type RHgX were determined in order to estimate the influence of a wide assortment of R structures and substituents on the ease of reduction of the mercury-anion bond. It is hoped that this information may suggest some correlation between the binding capacity of organic mercurials for proteins and the electronic influence of the organic moiety, possibly becoming a basis for the interpretation of their biological activity.

The compounds were studied in the supporting electrolyte, 0.1 *M* $NH_4Cl \cdot NH_4OH$, pH 7.4 being present mainly as the chloride species (2), except for 1,1′-oxalyldiiminobis (2-methoxy-trimethylenemercuri) bis-(2,5-dioxo-4-imidazolidineacetic acid, and 2,4-dioxo-3-imidazolidylmercuri-mercaptoacetic acid. In the former compound the mercury is bound to the nitrogen of the imidazolidine ring; in the latter compound the mercury is bound in a mercaptide.

None of these reductions is reversible; nevertheless, the waves are considered to be largely diffusion-controlled as judged by the normalcy of the polarograms at the low concentration levels employed (3) and the agreement between the depolarizing influence of the R groups and the polarographic stability.

METHODS AND MATERIALS

The mercurials studied are listed in Table I. Many were obtained as generous gifts from pharmaceutical houses[1] (PMOH) and phenylmercuric acetate (PMAC) were purchased from Berk and Co.; *p*-chloromercuribenzoate (PCMB) was obtained from Bios Laboratories, and *p*-tolylmercurichloride (PTMC), methylmercurichloride (MMC), and ethylmercurichloride (EMC) were purchased from Delta Chemical Works, Inc.

PMOH, PMAC, and PCMB were recrystallized before using. The other compounds were used as obtained. The purity of these compounds was judged to be sufficient for these studies from the data obtained with the samples.

Stock solutions of the mercurials were made at concentration level of $2 \times 10^{-4} M$. The mercurial was dissolved usually in water. In some cases (as with PCMB) it was necessary to dissolve it in a small amount of concentrated NH_4OH, neutralize with HCl, and dilute with supporting electrolyte solution to the desired concentration. Stock solutions were diluted with 0.2 *M* ($NH_4OH \cdot NH_4Cl$) before use, and the polarographic stability of the mercurial was observed at a concentration of $1 \times 10^{-4} M$. Possible decomposition of the solution was determined from a comparison of the polarograms of older solutions with those of a freshly prepared one. Where necessary, solutions were freshly prepared weekly.

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[1] Abbott Laboratories, Ciba Pharmaceutical Products, Inc., Lakeside Laboratories, Inc., Merck Sharp and Dohme Research Laboratories, Parke Davis and Co., The Squibb Institute, Sterling Winthrop Research Institute, The Upjohn Co., and Wyeth Laboratories, Inc.

The polarograms were obtained with an American optical recording electropolarizer model G-1 using a dropping mercury electrode. The drop time of the mercury electrode was 2.5 seconds per drop with a constant pressure of 16.5 cm. of mercury.

The temperature of the mercurial solutions was kept constant at 25° in a water bath. The solutions were deoxygenated by passing in oxygen-free nitrogen for 20 minutes. The solutions were allowed to equilibrate in the water bath for 10 minutes prior to the determinations.

RESULTS

Except for a few compounds—2-hydroxymercuri-3-methoxy-3-phenyl propanoic acid, N-(3-chloromercuri-2-piperidino-propyl) nicotinamide dihydrochloride, 1,1′-oxalyldiiminobis(2-methoxy-trimethylenemercuri)bis(2,5-dioxo-4-imidazolidineacetic acid), 2-acetomercuri-3-methoxy-butanoic acid, and 2,4-dioxo-3-imidazolidylmercuri-mercaptoacetic acid—which exhibited a single reductive wave, all of the mercurials listed in Table I were decomposed irreversibly in a two-step process. Benesch and Benesch (4) and later Wuggatzer and Cross (5) found that PCMB, phenylmercuric chloride (PMC), and phenylmercuric nitrate (PMN) are decomposed polarographically in two-electron steps, resulting in the formation of the organic mercurial radical in the first step and the reduction of this radical in the

TABLE I.—COMPOUNDS STUDIED POLAROGRAPHICALLY

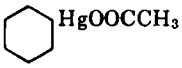
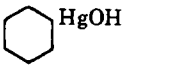
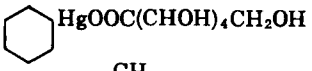
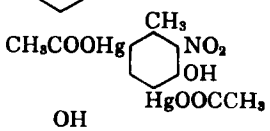
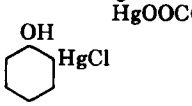
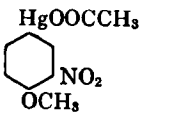
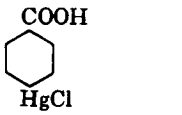
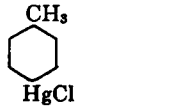
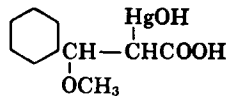
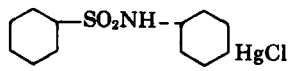
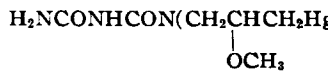
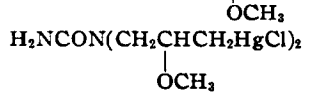
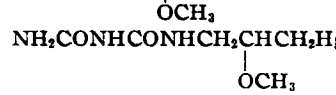
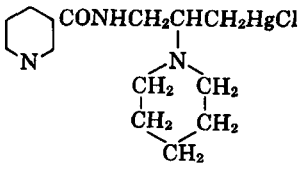
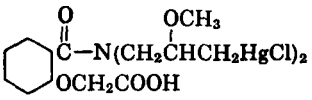
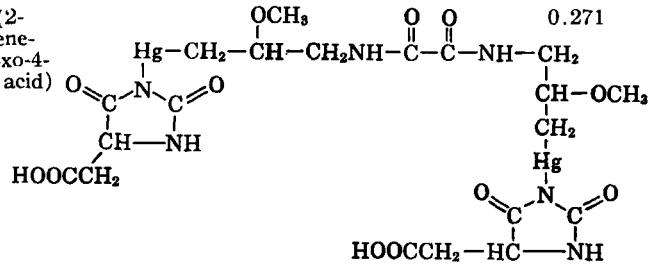
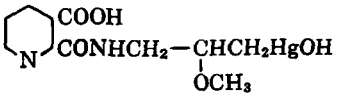
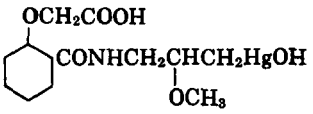
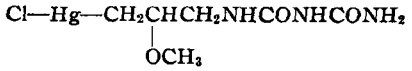
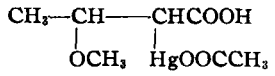
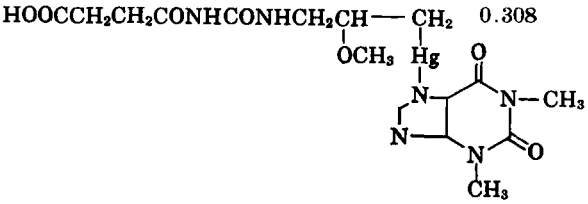
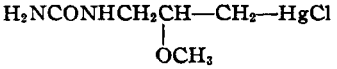
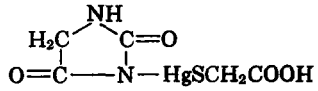
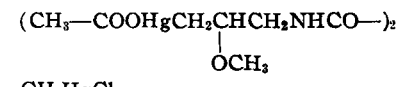
Compound Name	Formula	First Half-Wave Average, $-E_{1/2}$	Second Half-Wave Average, $-E_{1/2}$	Maxima Range, Volts
Acetomercuribenzene (phenylmercuric acetate, PMAC)		0.123	0.884	...
Hydroxymercuribenzene (phenylmercuric hydroxide, PMOH)		0.118	0.859	...
Glyconomercuribenzene		0.140	0.924	...
2-Nitro-3-hydroxy-4,6-diacetomercuritoluene		0.075	1.038	...
2-Chloromercuriphenol		0.131	0.838	...
1-Acetomercuri-3-nitro-4-methoxy-benzene		0.143	1.502	
4-Chloromercuribenzoic acid (*p*-chloromercuribenzoate, PCMB)		0.170	0.884	...
1-Methyl-4-chloromercuribenzene (*p*-tolylmercurichloride, PTMC)		0.178	0.923	...
2-Hydroxymercuri-3-methoxy-3-phenyl propanoic acid		0.196	...	...
1-Benzene sulfonamido-4-chloromercuribenzene		0.204	1.05	...
N,N-Bis(3-chloromercuri-2-methoxy-propyl)biuret		0.208	1.040	...
N,N-Bis(3-chloromercuri-2-methoxy-propyl) urea		0.216	0.958	...
N-(2-Methoxy-3-hydroxy-mercuri-propyl) biuret		0.235	0.864	...

TABLE I.—(*Continued*)

Compound Name	Formula	First Half-Wave Average, $-E_{1/2}$	Second Half-Wave Average, $-E_{1/2}$	Maxima Range, Volts
N-(3-Chloromercuri-2-piperidinopropyl) nicotinamide dihydrochloride		0.254	...	...
2-{N,N-Bis [3-(chloromercuri)-2-methoxypropyl] carbamyl-phenoxy} acetic acid		0.259	0.928	...
1,1′-Oxalyldiiminobis(2-methoxy-trimethylenemercuri)bis(2,5-dioxo-4-imidazolidineacetic acid)		0.271	0.900	...
2-(3-Hydroxymercuri-2-methoxy-propyl carbamyl) nicotinic acid		0.277	(1.02)	1.14 to 1.15
(Salyrganic acid) 2-(3-hydroxymercuri-2-methoxy-propyl) carbamyl-phenoxy acetic acid		0.279	(0.975)	1.06 to 1.08
1-[3-(Chloromercuri)-2-methoxy-propyl]biuret		0.283	(1.035)	1.15 to 1.17
2-Acetomercuri-3-methoxy-butanoic acid		0.285	...	...
Theophylline salt of N-(2-methoxy-3-hydroxy-mercuripropyl)-N′-succinyl urea[a]		0.308	0.882	...
N-(2-Methoxy-3-chloromercuripropyl) urea[b]		0.316	(0.975)	1.04 to 1.08
2,4-Dioxo-3-imidazolidyl-mercuri-mercaptoacetic acid		0.371	...	...
N,N′-Bis[3-(hydroxymercuri)-2-methoxy-propyl] oxamide, diacetate ester		0.372	(1.035)	1.09
Chloromercurimethane (methylmercurichloride, MMC)	CH_3HgCl	0.428	(1.30)	1.35 to 1.36
Chloromercuriethane (ethylmercurichloride, EMC)	CH_3CH_2HgCl	0.495	(1.39)	1.49 to 1.50

[a] Marketed as Mercurhydrin by Lakeside Laboratories. [b] Marketed as Neohydrin by Lakeside Laboratories.

second step to benzene and metallic mercury. This two-step reduction was also observed for a series of phenylmercuric amide and imide compounds (6).

It would be expected that the polarographic stability of PMOH, PMAC, and phenylmercuric glyconate should be identical because of the excess of chloride ion, and this is found to be true within ± 0.02 v. (see Table I). Taking this deviation as an allowable experimental error for all the determinations, it will be observed from the table that the R group of the mercurials has considerable influence on the half-wave potential. The phenyl ring compounds have the lowest and MMC and EMC the greatest negative $E_{1/2}$. Chain compounds with a β-methoxy group and biuret or urea in the chain have intermediate values. The entire range of voltage for the compounds varies from −0.127 v. for the phenyl compounds to −0.490 v. for EMC.

The direction of this change follows closely the depolarization effect of the R groups on the polarity of the mercury-chloride bond resulting in a change in the electron density of the mercury. Compounds in which mercury is attached directly to the benzene ring would be expected to have a higher polarity of the Hg-Cl bond, *i.e.*, a lower electron density on the mercury. The decomposition of these compounds at lower voltage agrees with the known polarizability of the benzene ring or its electronegativity to use Kharash's terminology (7).

The influence of substituents in the ring, on the other hand, seems to have relatively minor effects. The −E and −I effect of the *meta* nitro group is not shown, apparently being offset by the +E effect of the *p*-methoxy as in 1-acetomercuri-3-nitro-4-methoxy-benzene. Compound 2-nitro-3-hydroxy-4,6-diacetomercuritoluene having the same substituents in the ring has a lower decomposition potential. Possibly the electrophilic character of the two chloromercuri groups may be a factor. Compound 2-chloromercuriphenol shows no influence of an *ortho* phenolate ion. The influence of a *para* carboxyl ion in 4-chloromercuribenzoic acid (*p*-chloromercuribenzoate, PCMB) increases the half-wave potential significantly. The *para* methyl group in 1-methyl-4-chloromercuri-benzene (*p*-tolyl-mercurichloride, PTMC) and a substituted sulfonamide group in 1-benzene sulfonamide-4-chloromercuribenzene show considerable polarographic stabilizing influence.

Among the chain compounds the greater polarographic stability of chloromercurimethane (methylmercurichloride, MMC) and chloromercuriethane (ethylmercurichloride, EMC) suggests a large depolarization of the mercury-chloride bond. The influence of a β-methoxy and the other groups on the chain is shown by the lower decomposition potentials of the remaining compounds. Assuming a standard variation of ±0.02 v. compounds N-(3-chloromercuri-2-piperidinopropyl) nicotinamide dihydrochloride to 2-acetomercuri-3-methoxy-butanoic acid of Table I have the same decomposition voltage, −0.270 ± 0.02 v. Compound N-(3-chloromercuri-2-piperidinopropyl) nicotinamide dihydrochloride has a mercury attached to an aromatic pyridine ring with the substituted piperidine ring in the β-position to the mercury. The chain compounds of this intermediate group are all β-methoxy chain compounds which may account for their similarity of decomposition potential.

The decomposition potential, −0.373 v., of 2,4-dioxo-3-imidazolidylmercuri-mercaptoacetic acid is considerably higher than that of the group, −0.270 ± 0.02 v. This high value is obtained because the decomposition potential is that of a mercaptide in which the polarity of the Hg-S bond is less than the Hg-Cl bond (8).

Compound 2-acetomercuri-3-methoxy-butanoic acid is somewhat more stable than the group of methoxy compounds. Its decomposition potential is −0.285 v. It is interesting that the analog of this compound, 2-hydroxymercuri-3-methoxy-3-phenyl propanoic acid, in which the β-methyl is replaced with phenyl, is lower than the group −0.198 v. In both cases there is a carboxyl group bound to the same carbon as the Hg. The higher decomposition potential of 2-acetomercuri-3-methoxy-butanoic acid follows a depolarization effect of the carboxyl ion but a higher electronegativity of the phenyl ethyl group in 2-hydroxymercuri-3-methoxy-3-phenyl propanoic acid may also be a factor in lowering the potential.

Table I gives the decomposition potentials of the second wave for the aromatic ring compounds. Presumably at this potential the phenyl mercury radical is decomposed to benzene or a substituted benzene and mercury. Again using the results obtained with acetomercuribenzene (phenylmercuric acetate, PMAC), hydroxymercuribenzene (phenylmercuric hydroxide, PMOH), and glyconomercuribenzene as an estimate of the variations expected, the range of decomposition voltage obtained is −0.859 v. to −0.924 v. or −0.891 ± 0.03 v. It will be observed that only three of the compounds, 2-nitro-3-hydroxy-4,6-diacetomercuritoluene, 1-acetomercuri-3-nitro-4-methoxy-benzene, and 1-benzene sulfonamido-4-chloromercuribenzene, fall outside this range indicating that influences of substituents such as OH, COOH, or CH_3 are slight. From an electronegativity point of view, a small influence would be expected since the polarity of the mercury-carbon bond is less than that of the mercury-chloride bond. Compounds 2-nitro-3-hydroxy-4,6-diacetomercuritoluene and 1-acetomercuri-3-nitro-4-methoxy-benzene contain a nitro group in the ring and show unusually high polarographic stability. This strange effect is still in agreement with the idea of polarizability of groups when it is remembered that the nitro group is reduced under the conditions of these experiments to hydroxylamine in a four-electron step. This wave was observed at approximately −0.4 v. The +E effect of this reduced group would result in a decreased polarity of the carbon-mercury bond. This is in agreement with the greater negative half-wave potential of the compound.

A similar relationship was observed in a pH study of *o*-chloromercuriphenol. With increased pH there was an increased polarographic stability of the compound in a general correlation with the +E activity of the phenolate ion (see Table II). In this study a measure of the decomposition potential of the first wave gave anomalous results possibly due to bonding of the mercury to the *ortho* phenolate group. No significant change in the potentials of the first wave was observed over the entire pH range (5.07 to 10.23).

A similar relation between depolarization effects

TABLE II.—EFFECT OF pH ON SECOND HALF-WAVE OF *ortho*-CHLOROMERCURIPHENOL

pH	$-IIE_{1/2}$
5.07	0.608
5.98	0.763
6.70	0.776
6.70	0.840
7.73	0.796
8.71	0.884
10.23	0.946
10.23	0.888

and half-wave potentials was also observed in a study of the second decomposition of the chain compounds. The results of this study are given in Table I. Several of the chain compounds, 2-(3-hydroxymercuri-2-methoxy-propyl carbamyl) nicotinic acid; (salyrganic acid) 2-(3-hydroxymercuri-2-methoxypropyl) carbamyl-phenoxy acetic acid; 1-[3-(chloromercuri)-2-methoxy-propyl] biuret; N-(2-methoxy-3-chloromercuripropyl) urea; N,N'-bis-[3-(hydroxymercuri)-2-methoxypropyl] oxamide, diacetate ester; chloromercurimethane (methylmercurichloride, MMC), and chloromercuriethane(ethylmercurichloride, EMC), exhibited maxima with sloping plateaus at the second reduction. This made the estimation of their half-wave potentials difficult and inaccurate. The positions of the maxima are reported along with the approximate $E_{1/2}$. Undoubtedly, these abnormalities are due to adsorption and reactivity of the radicals but the position of the maximal wave is of some interest in terms of the polarization effects of the R groups. With PMOH as standard with a decomposition voltage of -0.891 ± 0.03 v., it will be observed that chain compounds N-(2-methoxy-3-hydroxymercuri-propyl) biuret; 2-{N,N-bis[3-(chloromercuri)-2-methoxy-propyl] carbamyl-phenoxy} acetic acid, and the theophylline salt of N-(2 - methoxy - 3 - hydroxy - mercuripropyl) - N'-succinyl urea are equally stable polarographically. These are β-methoxy chain compounds and the theophylline salt of N-(2-methoxy-3-hydroxy-mercuripropyl)-N'-succinyl urea has, in addition, an added theophylline ring. Compounds chloromercurimethane (methylmercurichloride, MMC) and chloromercuriethane (ethylmercurichloride, EMC) appear again as the most stable, correlating the +I effect of the methyl and ethyl chains, respectively. Compounds N,N-bis(3-chloromercuri-2-methoxy-propyl) biuret; N,N-bis(3-chloromercuri-2-methoxy-propyl) urea; 2-(3-hydroxymercuri-2-methoxy-propyl carbamyl) nicotinic acid; (salyrganic acid) 2-(3-hydroxymercuri-2-methoxy-propyl) carbamylphenoxy acetic acid; 1-[3-(chloromercuri)-2-methoxy-propyl] biruet; N-(2-methoxy-3-chloromercuripropyl) urea, and N,N'-bis[3-(hydroxymercuri)-2-methoxy-propyl] oxamide, diacetate ester are methoxy chain compounds and have an average decomposition potential of -0.983 ± 0.06 v.

As was stated before, 2-hydroxymercuri-3-methoxy-3-phenyl propanoic acid; N-(3-chloromercuri-2-piperidinopropyl) nicotinamide dihydrochloride; 1,1' - oxalyldiiminobis(2 - methoxy - trimethylenemercuri)bis(2,5-dioxo-imidazolidineacetic acid); 2-acetomercuri-3-methoxy-butanoic acid, and 2,4-dioxo-3-imidazolidylmercuri-mercaptoacetic acid appeared anomalous in that they exhibited a single reductive wave. Presumably this reduction may involve the addition of two electrons. An electronic interpretation would require that the two mercury bonds have the same polarity. This cannot be answered with the present data available, but it is interesting that in 2,4-dioxo-3-imidazolidylmercuri-mercaptoacetic acid the mercury is bonded to sulfur and nitrogen and in 1,1'-oxalyldiiminobis-(2-methoxy-trimethylenemercuri)bis(2,5-dioxo-4-imidazolidineacetic acid), to nitrogen and carbon. Possibly, proximate groups may also influence the polarity of the bonds.

DISCUSSION

The relation of the depolarizing influence of the R group structure with the polarographic stability of the compounds at the first wave is consistent in general with a mercury-chloride bond polarity of Hg^{d+}–Cl^{d-}. Those groups attracting electrons decrease the electron density on the mercury and those donating electrons decrease the polarity. Since the polarographic influence of the R group in the second reduction is in the same direction, an electronic interpretation would suggest that the polarity of the carbon-mercury radical bond is C^{d+}–Hg^{d-}. Radical formation does not occur apparently with all the compounds studied. In these cases there may be similar polarities in the two mercury bonds, as for instance in a compound in which the mercury is bonded to nitrogen and sulfur. This situation can be represented as N^{d-}–Hg^{d+}–S^{d-}.

At present, some organic mercurial compounds of the type

$$R—CH_2—\underset{\displaystyle OY}{\underset{|}{CH}}—CH_2—HgX$$

have found clinical usefulness as diuretics. While the X and Y groups have a small influence on activity, it is evident that the nature of the R group is fundamental (9). Compounds of the type R—Hg—X in which R is simply an aromatic ring or an aliphatic chain are not useful because of toxicity. It is interesting that the ring compounds of this type have low polarographic stability (see Table I) and those where R is a simple alkyl have large negative half-wave potentials. Among the intermediate compounds, polarographically speaking, are those which have found some clinical usefulness.

At present, it is impossible to correlate more closely toxicity or diuretic activity with half-wave potentials but this study suggests at least a possible basis for biological selectivity of the mercury compounds. From the demonstrated correlation between polarographic stability and polarizability of groups, it can be concluded that the R, X, and Y groups exert effects on the polarity of the mercury bonds and on the electron density around the mercury atom.

According to Boyer (2) the ease of dissociation of the mercury compound is apparently not of prime importance in biological activity. It seems from the work of Weiner and Müller (10) and others that the intact molecule is involved, at least in diuretic activity. If this is true, then possibly the electron density on the mercury plays the determining role in biological activity. This view is suggested by the rough correlation observed in the present work between polarographic stabilities and clinical usefulness

and by the fact that compounds of different polarographic stabilities exhibited correspondingly different inhibitory activities on trypsin proteolysis (work in progress).

This view of mercury activity proposes a mechanism by which organic groups may produce selectivity by controlling the electron density on the mercury moiety.

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A New Oral Gelatinized Sustained-Release Dosage Form

By NOBUO TANAKA, SETSUKO TAKINO, and ISAMU UTSUMI

A new sustained-release micropellet was prepared which takes advantage of the relationship of gelatin solubility to the hardness of the gelatin caused by formalin treatment. This new gelatin micropellet could be produced more easily than the coated micropellet employed in the usual sustained-release dosage forms.

THE SUSTAINED-RELEASE principle has universal application in the field of practical pharmacy. It is generally understood that gelatin is digested in the human gastrointestinal tract and that the rate of hydrolysis of the gelatin can be varied by hardening. This suggested that if medication was dissolved or suspended in a gelating sol and the gelatin treated with formalin-isopropanol, the rate of hydrolysis in the gastrointestinal tract would decrease. In this study, the relationship of the hydrolysis to the grade of gelatin hardening was applied to produce a sustained-release gelatin micropellet.

As described in the *Experimental* section, the technique utilized to produce the gelatin sustained-release micropellet is easier than the more complicated and painstaking process required to produce the conventional coated micropellet. The gelatin micropellet (containing medication) had a diameter of 0.3 to 0.5 mm. To obtain the desired sustained-release effect, the gelatin micropellet was stored in 10% formalin-isopropanol at 2–5° for varying periods of time. From the results of the *in vitro* dissolution test, the treated gelatin micropellet was found to prolong protease hydrolysis.

Urinary excretion, blood concentration, and biological kinetic theory data have been employed to evaluate oral sustained-release dosage forms and such studies have been reported recently in the literature (1–5). We used chemical determinations of the amount of medication in the blood or urine to examine the delay in absorption and urinary excretion of the medication imbedded in the treated gelatin micropellets. As gelatin can be digested in the gastrointestinal tracts of carnivorous and omnivorous animals, five dogs and three humans were used to evaluate the *in vivo* sustained-release of sulfanilamide (SA) and riboflavin (RF) through blood concentration determinations.

EXPERIMENTAL

Preparation of Dosage.—Table I shows the physical properties of gelatin and mineral oil used in the production of the micropellets.

An apparatus to produce the micropellets also is shown in Fig. 1. The apparatus consists of a stainless steel vessel, stirring wing, and motor. Two-hundred grams of water was added to 60 Gm. of gelatin. After the gelatin swelled 40 Gm. of SA powder (less than 50 μ in diameter) was added.

TABLE I.—PHYSICAL PROPERTIES OF GELATIN AND MINERAL OIL

	Viscosity, cps.	pH	M. p., ° C.	Sol-gel Transformation ° C.
Gelatin	23	6.70	30.5	25.7
		Sp. Gr.		Viscosity, cps.
Mineral Oil		0.853 (15.5° C.)		13.0 (35° C.) 22.0 (20° C.)

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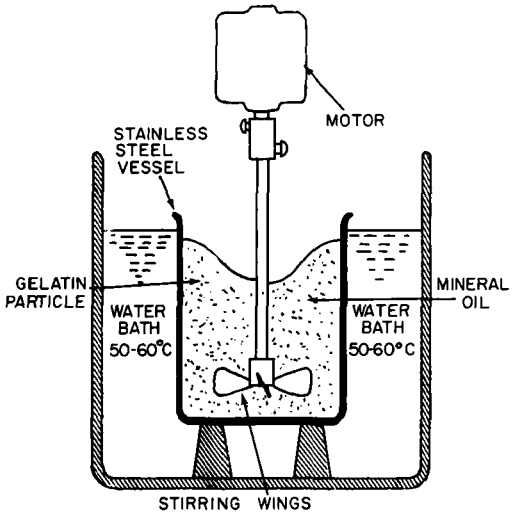

Fig. 1.—Apparatus used to produce gelatin micropellets.

TABLE II.—CHARACTERISTICS OF SA AND RF MICROPELLETS

Size	Yield, %	Content, %
SA		
Less than 24 mesh	7	38.08
24–35 Mesh	72	31.12
35–50 Mesh	17	20.32
More than 50 mesh	4	10.54
RF		
Less than 24 mesh	4	5.15
24–35 Mesh	81	5.10
35–50 Mesh	13	5.25
More than 50 mesh	2	5.30

The mixture was warmed on a steam bath to form a sol. Gelatin sol was poured into 600 Gm. of mineral oil heated previously to about 55–60° and stirred at 18–22 r.p.m. After 5 or 6 minutes, the vessel was steeped in ice water, cooled quickly to less than 5°, and kept at the same temperature until the gelatin microdrops became a perfect gel. After 300 Gm. of about 5° isopropanol was added to the vessel for dehydration, the solution was stirred about 5 minutes; then the gelatin micropellets were separated. The micropellets were then washed two times with 100 Gm. of 5° isopropanol and dried until the alcohol disappeared. The sustained-release effect of micropellets were obtained by immersing 1 Gm. of the micropellets into 10 ml. of 10% formalin-isopropanol in a covered vessel and hardened in a refrigerator at 2–5° for 24 hours and dried.

The method of RF was almost the same as with SA. One-hundred and seventy grams of water was added to 76 Gm. of gelatin. After the gelatin swelled, 4 Gm. of RF powder (less than 50 μ in diameter) was added, and the same procedure with SA was used. The hardening time of the micropellets was 24, 48, or 72 hours. These micropellets are shown in Tables II and III.

In Vitro Dissolution Experiment.—Micropellets equivalent to 100 mg. of SA and 200 ml. of simulated gastric fluid (dissolution medium) were put into a flask and immersed in thermostat adjusted to 37 ± 1°.

At appropriate intervals of time, 20 ml. was removed from the flask for analysis; a similar volume of dissolution medium was added each time to maintain constant volume. Five milliliters of 20% trichloroacetic acid was added to a 20-ml. quantity used for analysis.

Animal Experiment.—Five mongrel dogs weighing approximately 7 Kg. were fasted overnight. The mixture of micropellets and a piece of wet bread were given orally to the dogs. Blood samples of 2 ml. were then withdrawn from the vein after 1, 2, 4, 6, 7, and 24 hours, respectively. Dogs were used repeatedly with a rest period of more than 6 days for their complete recovery.

Method of Analysis for SA.—Tsuda's (6) modification of the Bratton and Marshall (7) method was used. The blood was hemolyzed by spraying into distilled water. Color was developed using 0.1% Tsuda reagent (β-diethylamino-ethylaminophthalene oxalate). Equivalent quantities (2 ml.) of blood were used in the preparation of the standard curve.

Method of Analysis for RF.—The method of analysis for RF was Yagi's method (8). Hemolyzed blood was decomposed with exposure to light. After acidifying by acetic acid, it was extracted with chloroform. Standard lumiflavin solution was prepared using 1 mcg./ml. standard RF solution instead of blood.

RESULTS AND DISCUSSION

As shown in Table IV, it is obvious from the results on various samples *in vitro* that hardened

TABLE III.—DOSAGES AND CONTENTS OF SA AND RF MICROPELLETS

Sample	Dosage and Contents
I	Gelatin micropellet containing 33.2% SA
II	Micropellet, treated for 24 hr., and containing 19.0% SA
III	RF-phosphate solution
IV	Gelatin micropellet containing 5% RF
V	Micropellet, treated for 24 hr., and containing 5% RF
VI	Micropellet, treated for 48 hr., and containing 5% RF
VII	Micropellet, treated for 72 hr., and containing 5% RF

TABLE IV.—PERCENTAGE OF ACCUMULATIVE SA RECOVERED IN THE *In Vitro* DISSOLUTION TEST

Sample I		Sample II	
Time	%	Time	%
5 min.	32.9	5 min.	5.9
10 min.	59.5	10 min.	9.9
15 min.	76.5	20 min.	30.7
35 min.	80.0	30 min.	39.6
1 hr.	89.6	45 min.	53.4
2 hr.	99.5	1 hr.	61.5
3 hr.	99.7	2 hr.	82.0
		3 hr.	86.0
		5 hr.	91.6
		7 hr.	94.1
		23 hr.	98.2
		30 hr.	99.6

TABLE V.—TOTAL SA CONCENTRATION IN BLOOD (MG.%). DATA FOR FIVE DOGS AFTER ORAL ADMINISTRATION OF SAMPLE I (250 MG. OF SA PER 1 KG. OF BODY WEIGHT)

Time, Hr.	Dog 1	2	3	4	5	Av.
2	6.56	6.44	6.17	6.08	6.00	6.27
4	13.88	10.20	8.77	8.78	9.62	10.25
6	9.21	9.41	9.35	9.16	9.62	9.35
7.5	7.63	8.38	7.94	7.47	8.53	7.99
16	2.00	2.93	2.24	1.83	5.25	2.95
20	2.25	1.77	1.22	1.05	3.11	1.88
24	1.15	1.16	1.03	0.08	1.76	1.18
30	0.84	0.62	0.46	0.34	0.64	0.58

TABLE VI.— TOTAL SA CONCENTRATION IN BLOOD (MG.%). DATA FOR FIVE DOGS AFTER ORAL ADMINISTRATION OF SAMPLE II (250 MG. OF SA PER 1 KG. OF BODY WEIGHT)

Time, hr.	Dog 1	2	3	4	5	Av.
2	0.47	0.49	0.45	0.43	0.51	0.47
4	1.56	1.85	1.43	1.13	1.68	1.59
6	3.73	3.69	4.01	3.88	4.14	3.84
8	6.35	5.07	5.86	6.65	4.77	5.77
10	7.00	7.42	7.13	8.29	7.46	7.43
16	7.09	9.88	10.00	9.35	11.03	9.46
18	8.00	9.10	10.78	9.35	8.22	9.09
20	9.14	8.53	9.14	6.44	8.15	8.08
22	8.60	8.34	8.99	8.00	7.87	8.36
24	8.57	7.99	8.81	7.46	6.54	7.87
25	7.90	7.26	7.87	7.01	5.01	7.01
28	6.25	6.89	6.75	6.67	4.14	6.68
30	6.42	5.73	6.73	5.99	3.03	5.58

micropellets have the prolongation of hydrolysis against protease in the simulated gastric fluid. In the RF micropellets, RF could not be measured by the same method as the *in vitro* test of SA micropellets because of the slight solubility of RF in simulated gastric fluid.

Dogs were given Sample I and Sample II orally at a dose of 250 mg. of SA per Kg. of body weight, and SA concentrations in blood were measured at the times shown. Blood concentration of SA reached a peak 3–4 hours after administration of Sample I. On the other hand, in Sample II maximum peak of SA concentration in blood appeared after 16

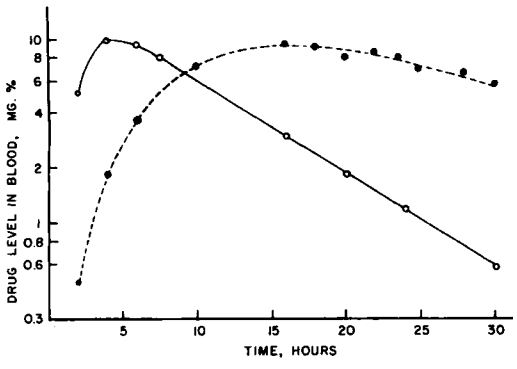

Fig. 2.—Logarithm of SA levels in blood against time after administration of SA gelatin micropellets to dogs. —O—O—, untreated micropellets; —●—●—●—, micropellets treated with formalin-isopropanol for 24 hours.

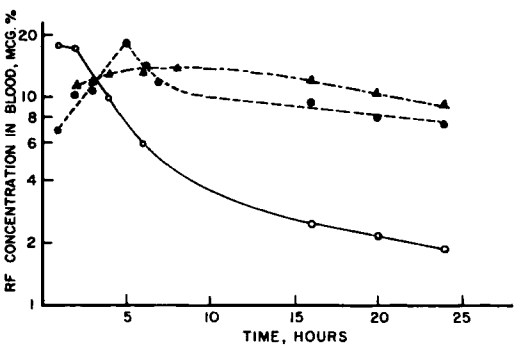

Fig. 3.—Logarithm of RF concentration in blood after administration of RF solution and its gelatin micropellets to dogs. —O—O—, RF solution; —●—●—●—, untreated micropellets; —▲—▲—▲— micropellets treated with formalin-isopropanol for 24 hours.

hours. Since the blood concentration of Sample II decreased more gradually than that of Sample I, it is certain that Sample II fulfills prolongation. The results of these experiments appear in Tables V and VI.

The decline of blood concentration of SA and its homologs after they had reached a maximum concentration, followed a pseudo first-order rate (5,9,10). To examine whether samples stayed in the digestive tracts, the logarithm of SA concentration in blood was plotted as a function of time (Fig. 2). A straight-line relationship of decrease resulted in Sample I after the maximum blood concentration. The possible interpretation is that Sample I almost dissolved in the gastrointestinal tracts in 3–4 hours. Sample II is considered to stay in the digestive tract for a long time since blood concentration increased until the 16th hour and decreased slowly after this. Judging from the above results, it is obvious that

TABLE VII.—TOTAL RF CONCENTRATION IN BLOOD (MCG.%). DATA FOR FIVE DOGS AFTER ORAL ADMINISTRATION OF SAMPLE III (1 MG. OF RF PER KG. OF BODY WEIGHT)

Time, Hr.	Sample III, mcg. % 1	2	3	4	5	Av.
1	17.5	16.0	19.0	18.7	18.8	18.0
2	17.0	15.5	18.1	18.1	18.8	17.5
4	9.8	9.2	11.1	10.7	9.2	10.0
6	5.8	5.3	7.2	7.2	4.5	6.0
16	2.5	2.2	2.6	2.5	2.7	2.5
20	2.3	2.0	2.4	2.0	2.3	2.2
24	1.8	1.9	1.5	1.9	2.4	1.9

TABLE VIII.—TOTAL RF CONCENTRATION IN BLOOD (MCG.%). DATA FOR FIVE DOGS AFTER ORAL ADMINISTRATION OF SAMPLE IV (1 MG. OF RF PER KG. OF BODY WEIGHT)

Time, hr.	Sample IV, mcg. % 1	2	3	4	5	Av.
1	5.0	4.0	8.4	9.2	8.4	7.0
2	10.5	9.3	11.2	12.0	9.5	10.5
3	10.9	9.7	11.2	12.4	10.8	11.0
5	17.0	15.3	18.5	19.7	22.0	18.5
6	9.8	16.0	15.1	13.7	15.3	14.0
7	8.7	15.2	13.3	12.0	10.8	12.0
16	8.8	9.6	11.3	8.8	9.0	9.5
20	6.2	7.6	10.0	8.3	7.9	8.0
24	6.8	7.5	7.4	7.0	8.8	7.

TABLE IX.—TOTAL RF BLOOD CONCENTRATION OF FIVE DOGS AFTER ORAL ADMINISTRATION OF SAMPLE V (1 MG. OF RF PER KG. OF BODY WEIGHT)

Time, hr.	Sample V, mcg. % 1	2	3	4	5	Av.
2	12.0	9.0	11.0	10.3	15.2	11.5
3	12.7	10.5	13.1	12.9	10.8	12.0
4	13.2	12.9	13.6	13.0	13.3	13.2
6	13.3	13.9	14.3	11.0	13.5	13.2
8	13.3	12.1	15.9	14.6	14.1	14.0
16	11.9	10.8	13.4	12.2	12.7	12.2
20	10.5	9.3	12.0	8.7	13.5	10.8
24	9.0	8.7	10.2	9.5	10.6	9.6

TABLE X.—THE RELATION OF RF QUANTITY (MCG.) EXCRETED IN URINE TO HOURS OF FORMALIN TREATMENT IN THREE ADULT HUMANS

Subjects→		A	B	C
Sex→		Man	Man	Woman
Age→		35	20	25
Sample	Collection Time, hr.	Subjects A	B	C
III	0–2	1092	1853	4208
	2–4	511	689	1867
	4–6	87	112	335
	6 to 7.5	57	91	221
IV	0–2	595	836	1689
	2–4	825	1071	2426
	4–6	161	199	509
	6 to 7.5	32	51	76
V	0–2	243	408	661
	2–4	884	1749	3142
	4–6	250	555	1098
	6 to 7.5	142	216	538
VI	0–2	113	216	274
	2–4	754	1168	2400
	4–6	368	595	1062
	6 to 7.5	183	255	644
VII	0–2	43	94	150
	2–4	463	760	1129
	4–6	339	520	1003
	6 to 7.5	205	362	950

gelatin micropellets hardened with formalin produce a sustained-release effect.

Both medication transport into blood and excretion in urine were determined on RF gelatin micropellets; the urinary excretion of RF is quicker than that of SA. Using the same dogs that were used in the experiment of SA micropellets, 1 mg. of RF per Kg. of body weight was administered to each dog and RF concentration in blood was determined. The results are shown in Fig. 3 and Tables VII–IX.

When Sample III was given to dogs (Fig. 3), the blood concentration gave a maximum level within 1 hour and then dropped quickly.

With Sample IV, RF concentration in blood reached a peak at about 4 hours; even 24 hours after dosing, RF concentration was higher than that of Sample III. In Sample V, a gradual rise occurred through 4 hours, reached a plateau and remained there for 3 hours, and 24 hours after

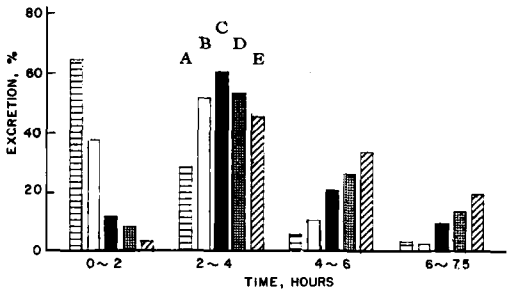

Fig. 4.—Ratio of RF in urine against time (after oral administration to three humans) if amount of accumulative RF excreted into urine for 7.5 hours is presumed 100%. A, RF solution, B, no treatment micropellets, C, micropellets treated for 24 hours, D, micropellets treated for 48 hours, E, micropellets treated for 72 hours.

dosing dropped to a blood concentration similar to Sample IV. Then the behavior of urinary excretion of RF in the load test on humans was examined. Results similar to those of the dogs were expected. The results are shown in Table X and Fig. 4.

When Sample III was administered, RF concentration in urine had a maximum value after 2 hours, but in other cases maximum excretion was shown about 4 hours after dosing. Table X shows that the recovered quantities of Samples VI and VII are lower than those of the other samples. This phenomena is probably due to the delay of excretion since the excreted quantities from 6 to 7.5 hours after administration rose proportionally to the time of treatment by formalin.

In these experiments there was a distinct difference in blood concentration response among Sample III, Sample IV, and Sample V. In the urinary excretion test, peak level of Sample II was observed 2 hours after dosing, while those of the other three samples appeared after 4 hours. Therefore, the sustained release of a certain drug cannot always be determined only by the peak of the urinary excretion, since the quantity excreted in urine is not directly related to blood concentration.

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Kinetics of Steroid Effects on Ca^{47} Dynamics in Dogs with the Analog Computer II

By EDWARD R. GARRETT, RICHARD L. JOHNSTON, and ELLIOTT J. COLLINS†

The effect of adrenal steroids on the distribution of intravenously administered Ca^{47} has been studied further by use of an analog computer. A distributive model, consistent with the literature, has been programmed on the computer and has been shown to be in agreement with the fecal and blood serum levels of Ca^{47}. Adrenal steroid effects can be assigned to a decrease in the calcium capacity of the metabolic pool with respect to the calcium capacity of the blood and to enhanced transfer of calcium to the gastrointestinal tract. This distributive model has been correlated with the empirical equations computer-derived for fitting the Ca^{47} data. Blood levels and fecal excretion of Ca^{47} are enhanced by steroid and depressed on recovery. It has been definitely proved that when the quasi equilibria of radioactive calcium among blood, soft tissues, metabolic pool, and contents of the gastrointestinal tract have been achieved, the relatively slow fecal elimination of Ca^{47} at an apparently linear rate is doubled by the steroid administered and relapses to the control values on recovery. The phenomena are consistent with the hypothesis that adrenal steroids not only inhibit the entry of endogenous calcium into the bone and enhance its secretion into the gastrointestinal tract but also promote the resorption of calcium from bone.

THE PREVIOUS PAPER in this series (1) considered the kinetics of adrenal steroid effects in four dogs and fitted the obtained values of intravenously administered Ca^{47} in blood, feces, and urine as functions of time with the analog computer.

It had been observed that steroid regimen increased the amount of Ca^{47} accumulated in the feces and urine with respect to the amounts of a prior control or presteroid period, and that this increase was reversed subsequently on cessation of steroid dosage (1, 2). It had also been observed that Ca^{47} blood levels after intravenous administration of Ca^{47} were generally higher during the steroid regimen than during the prior control period (1, 2).

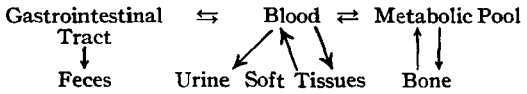
On recovery from steroid administration, a decreased rate in the resorption of Ca^{47} (possibly from the bone) was reflected in the long term fecal and urinary excretion of the radioisotope (1). Unfortunately, long term fecal and urinary excretion studies of Ca^{47} had not been conducted during the prior control periods.

The dogs in these previous studies (1) were relatively unmatched, were treated with antibiotics, and were biopsied. This chemotherapy and possible surgical shock may have had an indeterminate effect on the studies and their results.

In light of these circumstances it was believed that further detailed investigation was warranted to eliminate possible perturbing influences such as antibiotics or surgery, to take full advantage of the knowledge that prolonged studies of Ca^{47} fecal excretion may give insight into the effects of adrenal steroids on the resorption of calcium from bone, to use animals as "matched" as possible to evaluate the consistency of effects in control, steroid, and recovery phases, and to study induced osteoporosis in the fast growth period of the immature dog.

This paper reports on these investigations and utilizes the analog computer to quantify the blood levels, fecal, and urinary amounts of Ca^{47} as functions of time in accord with the method previously established (1). In addition, analog computer methods and techniques are used to fit a more realistic model for the compartmental transferences of Ca^{47}.

Such a model, variously modified, has been considered by several authors (3–7):



(Eq. 1)

It must be well understood that curve or data fitting with time can be accomplished not only by the model of Eq. 1 but also by kinetically equivalent models (3). For example, the transfer of calcium from the blood to the metabolic pool as mediated through the soft tissues may also fit the data. The amount of Ca^{47} excreted in the urine by the dog is small and would make a

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TABLE I.—TABULATION OF CONSTANTS[a] FOR ADDITIVE SERIES OF EXPONENTIALS, $\Sigma_i A_i e^{-k_i t}$, CHARACTERIZING THE DECREASE OF Ca^{47} FROM THE BLOOD AFTER INTRAVENOUS ADMINISTRATION[b]

Study[c]	Dog 1 A	Dog 1 B	Dog 1 C	Dog 2 A	Dog 2 B	Dog 2 C
A_1	0.0404	0.0350	0.0298	0.0356	0.0266	0.0405
A_2	0.0177	0.0158	0.0143	0.0175	0.0097	0.0124
A_3	0.0073	0.0094	0.0088	0.0073	0.0108	0.0109
A_4	0.0019	0.0029	0.0031	0.0017	0.0032	0.0030
$\Sigma_i A_i$	0.0673	0.0631	0.0560	0.0621	0.0503	0.0668
$\Sigma_i A_i - A_1$	0.0269	0.0281	0.0262	0.0265	0.0237	0.0263
$10^3 k_1$	3.77	2.00	3.10	2.68	2.88	3.49
$10^4 k_2$	2.77	1.63	1.82	2.04	3.13	1.90
$10^5 k_3$	3.50	2.12	2.34	3.50	2.66	2.73
$10^6 k_4$	5.06	4.06	4.75	5.06	4.49	4.83
Study[c]	**Dog 3 A**	**Dog 3 B**	**Dog 3 C**	**Dog 4 A**	**Dog 4 B**	**Dog 4 C**
A_1	0.0530	0.0415	0.0395	0.0530	0.0595	0.0450
A_2	0.0199	0.0151	0.0200	0.0199	0.0200	0.0200
A_3	0.0079	0.0186	0.0100	0.0087	0.0100	0.0054
A_4	0.0021	0.0031	0.0032	0.0022	0.0038	0.0035
$\Sigma_i A_i$	0.0829	0.0783	0.0727	0.0838	0.0933	0.0739
$\Sigma_i A_i - A_1$	0.0299	0.0368	0.0332	0.0308	0.0338	0.0289
$10^3 k_1$	3.36	2.07	5.78	3.37	2.61	2.22
$10^4 k_2$	2.30	1.89	2.32	1.96	1.40	0.97
$10^5 k_3$	3.46	4.97	2.56	3.47	1.50	1.62
$10^6 k_4$	5.17	4.34	4.71	5.06	4.75	6.59

[a] A_i are in per cent of initial total dose of Ca^{47}/ml. of serum and k_i are in sec.$^{-1}$ [b] Study A was the control study; Study B was during 2 mg./Kg./day steroid administration; the recovery Study C was conducted after cessation of steroid administration. [c] The injected doses of Ca^{47} were in c.p.m.–11.01 × 10^6 for A, 8.11 × 10^6 for B, and 14.3 × 10^6 for C.

negligible contribution to the overall balance of compartments and rates.

Reversible first-order expressions for Ca^{47} transfer between gastrointestinal tract and blood, between metabolic pool and blood, and between metabolic pool and bone are gross oversimplifications of complex physiological phenomena. However, such a model as Eq. 1 is most certainly a closer fit to reality than a linear equation of exponentials (1, 8–13).

The apparent first-order rate constants, k_i, which can be derived from analog computer fitting of the Ca^{47} data have an alternative interpretation as r/Ca_i, the fractional amount of calcium Ca_i renewed or turned over in a unit time where r is the turnover rate of calcium, amount/unit time, renewed in the compartment (see *Appendix*) (14, 15).

EXPERIMENTAL AND METHODS

Treatment of Animals.—Four beagles were used in these experiments. The animals were conditioned to the metabolism cages and laboratory routine prior to experimentation. Pertinent data for Dogs 1–4 given as initial age in days and sex are for Dog 1, 227 (F); Dog 2, 222 (F); Dog 3, 213 (M); Dog 4, 213 (M). The methods and procedures of intra-

TABLE II.—CONSTANTS FOR CHARACTERIZATION OF ACCUMULATION OF Ca^{47} IN DOG FECES AND URINE FROM (1)[a] $\Sigma P_i = P_\infty(1 - e^{-kt})$ AND (2)[b] $\Sigma P_i = mt + b$

Dog	Study[c]	Feces P_∞	Feces 10^6k	Feces m	Feces $\%Ca^{47}_{Bone}$[d]	Feces 10^4R[e]	Feces b	Urine P_∞	Urine 10^6k	Urine m	Urine 10^6R[e]	Urine b
1	A	9.6	3.12	0.0129	90	1.43	6.30	0.41[f]	5.88	0.00065	7.22	0.303
	B	33.2	3.05	0.0141	66.1	2.13	28.2	0.70	6.24	0.00115	17.4	0.535
	C	21.0	3.65	0.0088	78.5	1.12	17.6	0.44	9.52	0.00031	3.94	0.392
2	A	9.6	4.46	0.0136	89.9	1.51	6.90	0.48[f]	6.50	0.00048	5.34	0.373
	B	32.6	4.7	0.0155	66.5	2.33	30.3	0.84	8.71	0.00113	16.9	0.700
	C	25.1	5.76	0.0101	74.3	1.35	23.4	0.52	10.89	0.00034	4.57	0.456
3	A	10.8	3.88	0.0114	88	1.30	8.30	1.22[f]	3.52	0.00105	11.9	0.96
	B	29.6	4.27	0.0203	69.1	2.93	24.8	1.33	5.1	0.00137	19.8	1.06
	C	17.0	5.78	0.0092	82.5	1.11	15.7	0.54	6.77	0.00039	4.72	0.44
4	A	11.2	2.94	0.0116	87.9	1.32	7.95	0.91[f]	5.20	0.00073	8.3	0.77
	B	29.0	4.8	0.0230	69.8	3.29	24.6	1.19	4.8	0.00100	14.3	0.93
	C	16.5	3.79	0.0097	82.3	1.17	13.5	1.16	5.97	0.00041	4.98	1.03

[a] Where ΣP_i is expressed in terms of per cent total dose and k in sec.$^{-1}$ [b] This is the deviation from the first-order accumulation of Ca^{47}, *i.e.*, P_∞ where m is in per cent total dose/hour and t is in hours. [c] The injected doses of Ca^{47} were in c.p.m.–11.01 × 10^6 for Study A, 8.11 × 10^6 for Study B, 14.3 × 10^6 for Study C, for all dogs. [d] This column relates to per cent of the total dose as Ca^{47} remaining in bone at *ca.* 200 hours and as can be estimated from 100 $-P_\infty$(urine) $- P_\infty$(feces). [e] $R = m/\%\ Ca^{47}_{Bone}$ is an estimate of the resorption rate adjusted for the Ca^{47} content of the bone where $\%\ Ca^{47}_{Bone}$ is defined in *footnote f*. [f] An anomalously high count was obtained for the first urine samples during the control phases that did not appear for other phases or for the same control phase for other studies. Plots of Ca^{47} accumulated in urine for runs A *vs.* time showed an intercept value at zero time. A technological bias is implied. *Ignoring of this first value of the urine for* the control phase, runs A, brought the urine data into the same pattern as the fecal data. These first urine Ca^{47} values in control phase A which were ignored were (in terms of per cent of total Ca_0^{47} dose at zero time): Dog 1, 0.22%; Dog 2, 0.38%; Dog 3, 0.65%; Dog 4, 0.28%.

TABLE III.—ESTIMATED SECOND LINEAR RATE,[a] R' OF RESORPTION OF Ca^{47} FROM BONE DURING RECOVERY PHASE, STUDY C, AFTER CESSATION OF STEROID ADMINISTRATION

Dog	m'[a]	b'	$10^4R'$	t', Hrs.
1	0.0146	22.5	1.89	800
2	0.0117	30.5	1.67	690
3	0.0127	22.5	1.69	760
4	0.0115	20.5	1.45	750

[a] $R' = m'/\%\ Ca^{47}_{Bone}$ is an estimate of the second linear Ca^{47} fecal excretion rate with time after t' hours adjusted for the $\%\ Ca^{47}_{Bone}$ content presumed to be in bone. The $\%\ Ca^{47}_{Bone}$ value is derived from $100 - b'$ at t' hours, *i.e.*, the per cent of the original Ca^{47} dose not excreted in the feces at that time where $b' = \Sigma P_i$ at $t = t'$. The slope, m', is in per cent of total dose/hour of the cumulative Ca^{47} (per cent of original Ca^{47} dose), *i.e.*, excreted in the feces, *i.e.*, ΣP_i after $t = t'$ and $\Sigma P_i = m'(t - t') + b'$. At t', *ca.* 700 hours, a sharp discontinuity in the linear fecal excretion of Ca^{47} observed after 200 hours did occur and these data describe the properties of this new linear relation of the cumulative Ca^{47} excretion, ΣP_i, in the feces with time.

venous administration of Ca^{47} and of the determination of radioactivity in blood serum, urine, and feces have been detailed elsewhere (2).

The studies were conducted in three sequential phases. At the ages cited above 50 μc. of Ca^{47} were administered (control, Study *A*), and the Ca^{47} distribution was studied with respect to time. At "Day 20" after the initial administration of Ca^{47}, 2 mg./Kg./day of a typical corticoid, 6-α-methylprednisolone was administered orally and Ca^{47} was again intravenously injected at "Day 41" and its distribution studied with time (steroid, Study *B*). At "Day 61," the steroid regimen was reduced for each dog at the rate of 1 mg./day so that at "Day 81," steroid administration was ceased. At "Day 131," Ca^{47} was again administered and distribution studied with time (recovery, Study *C*).

The times of blood sampling and urine and feces collection after Ca^{47} administration were as previously given (1), except the feces and urine collections were prolonged for 25 days after Ca^{47} administration during the control and steroid studies and for 58 days during the recovery studies. No antibiotics were administered or biopsies were performed.

Treatment of Data.—The procedures used for calculating and treating the data from the blood serum, urine, and feces were given in detail in the previous publication (1). Sufficient sample was taken and time of counting used so that all counting error was less than 2%. As previously discussed, values were corrected for normal decay from the data obtained by the simultaneous counting of an aliquot of the initial Ca^{47} solution used for injection.

Curve Fitting and the Analog Computer.—The disappearance of labeled Ca^{47} from the blood after intravenous administration can be represented by a four-factor linear homogeneous equation of exponentials (1, 8–13)

$$Ca_B{}^{47} = 100\ Ca^{47}/Ca_0{}^{47} = \Sigma_i A_i e^{-k_i t} = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + A_3 e^{-k_3 t} + A_4 e^{-k_4 t} \quad \text{(Eq. 2)}$$

where Ca^{47} is the amount of Ca^{47} per ml. of blood

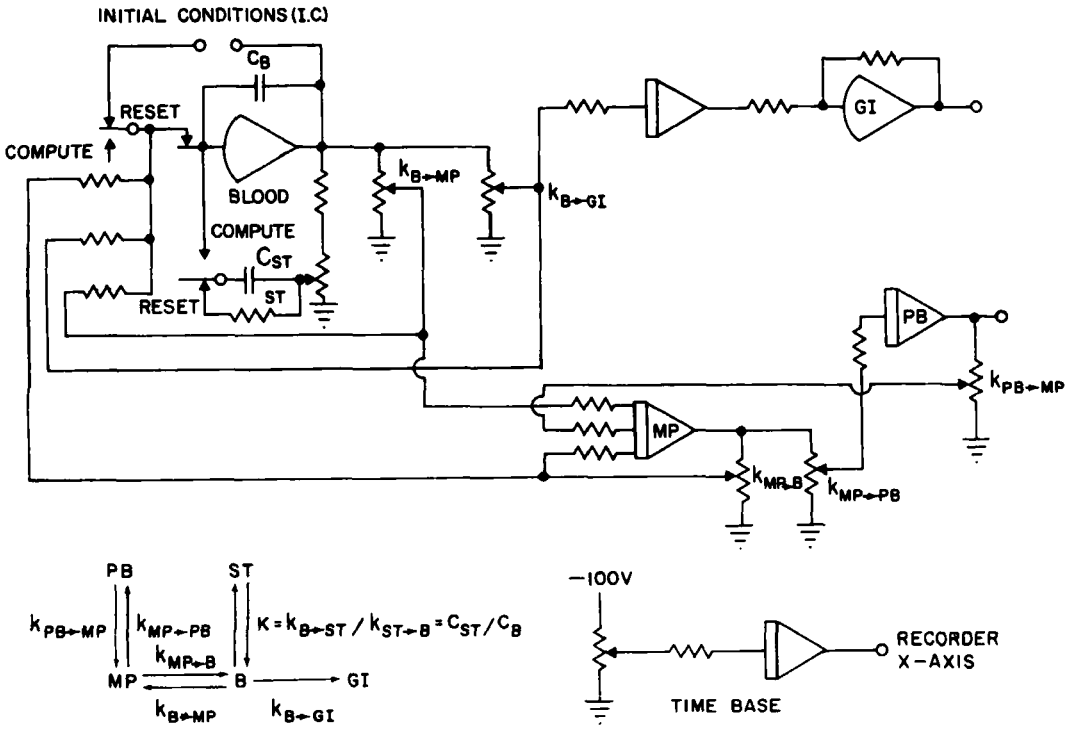

Fig. 1.—The electronic dog. Schemata for the programming of the analog computer for Ca^{47} data in the blood and feces up to 240 hours. The k values represent the respective potentiometers that are varied to obtain the rate constants, the sequence of their respective subscripts represent the directions of transfer of the Ca^{47}. The symbols, *B* (blood), *ST* (soft tissues), *MP* (metabolic pool), *PB* (bone), and *GI* (gastrointestinal tract) represent the various possible depots for Ca^{47} in the animal. The C_{ST} and C_B represent the capacitors for the soft tissue-blood equilibrium.

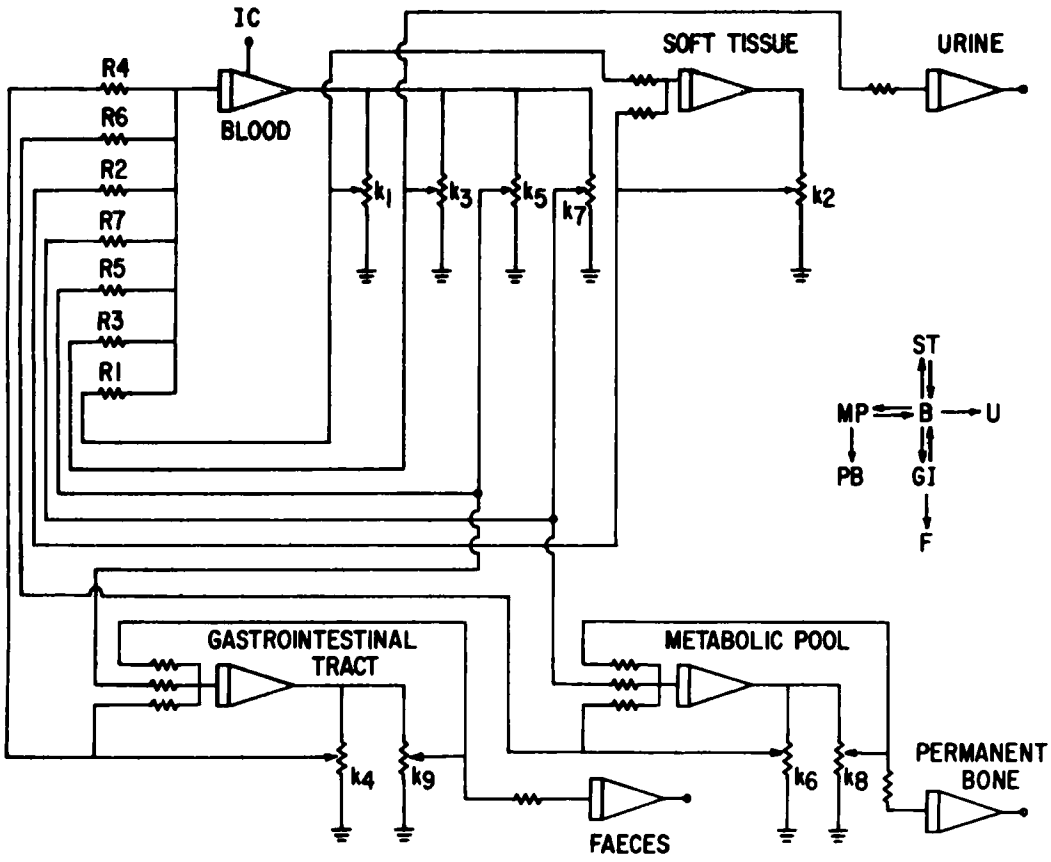

Fig. 2.—The electronic dog. A more complete schemata for the programming of the analog computer and used in part to fit Ca^{47} data up to 11 hours.

serum at any time, t, and Ca_0^{47} is the initial dose, so that Ca_B^{47} is the per cent of total dose administered/ml. of serum.

The significance and limitations of this expression as a reflection of reality and the methods of determination of A_i and k_i by analog and digital computer methods have been previously described (1).

The A_i and k_i values for these studies of four dogs during a control phase A, a steroid regimen, B, and a recovery phase, C, are given in Table I.

The parameters of the curve fitting of the fecal and urinary data by the analog computer are given in Tables II and III in accord with the methods and developments previously given (1).

The Electronic Dog and the Analog Computer

Programming of the Chosen Model for Ca^{47} Distribution.—The basic philosophy of evaluating the consistency of a physiological model with experimental data by means of simulation with an analog computer has been given previously (14). The analog computer setup as finally programmed for all data in excess of 700 minutes is given in Fig. 1. The output voltage of each integrator represents the time variable amount (concentration times a volume factor) of Ca^{47} distributed to a particular portion of the dog's anatomy on the basis of the model chosen, *i.e.*, blood (B), gastrointestinal tract (GI), metabolic pool (MP), and permanent bone (PB).

The integrators are connected by rate setting potentiometers in a conventional manner to simulate the distribution of Ca^{47} on the basis of the chosen model.

The amount of the dosage is represented by the initial condition voltage (IC) applied to the blood

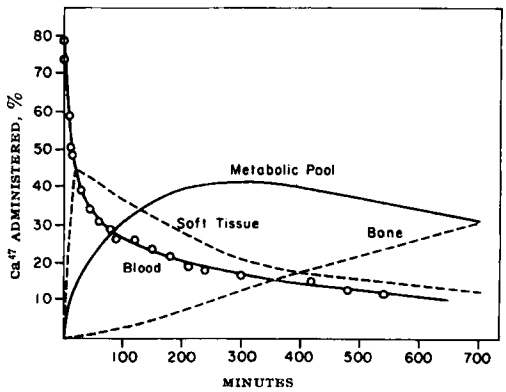

Fig. 3.—Ca^{47} as per cent of intravenously administered dose in the various depots of Dog 1, steroid phase B *vs.* time up to 700 minutes on the basis of a good fit to the plotted blood serum data. The other plots are the "read-out" of the analog computer in accordance with the program of Fig. 2.

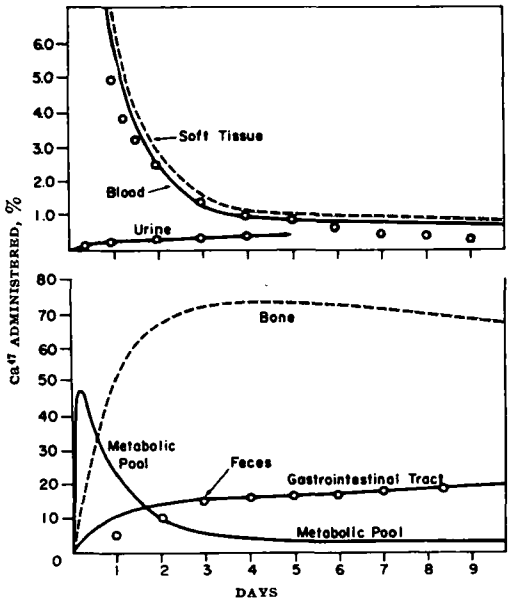

Fig. 4.—Ca^{47} as per cent of intravenously administered dose in the various depots of Dog 1, steroid phase *B vs.* time up to 9 days on the basis of a good fit to the plotted blood serum data and the latter values in the feces. The other plots are the "read-out" of the analog computer in accordance with the program of Fig. 1. A portion of the urine data is plotted for comparison.

(*B*) integrator since the method of administration of Ca^{47} was intravenous.

An additional integrator generates a voltage proportional to time as required by the X-Y recorder used, Fig. 1. The time scale was chosen so that for the 700-minute curves of Figs. 3 and 5, 1 minute of machine time equaled 10^3 minutes of real time; for the 10-day curve of Figs. 4 and 6, 1 minute of machine time equaled 10^4 minutes of real time.

Originally, the more elegant computer program given in Fig. 2 was used in fitting the data. However, drift or leakage resulted over the entire time interval of 10 days with the large number of integrators and with apparent rate constants which varied over four powers of ten. A more expensive computer comprised of precision components (resistors and capacitors) would have been able to simulate without drift. However, with the equipment at hand it was necessary to improvise.

The rate of decrease of Ca^{47} in the blood can be fitted to a linear sum of exponentials and the k_1 value of the first of these, $A_1e^{-k_1t}$, representative of the rapid equilibration between blood and soft tissues, is relatively of high magnitude. The residual exponentials contribute little to Ca^{47} decrease in the blood while the first exponential is operative. Conversely, the equilibration between blood and soft tissue at times in excess of 100 minutes can be considered as instantaneous with respect to the other distributive factors that are rate determining in the loss of Ca^{47} from the blood.

Thus a valid simplification of the program of Fig. 2 in excess of 100 minutes is the removal of the integrator representing the soft tissue (*ST*) and substitution of equilibrating capacitors as in Fig. 1 to account for this rapid equilibration of blood Ca^{47} with soft tissue. The model used can be considered as liquid instantaneously seeking the same level in joined compartments of capacities proportional to the apparent compartmental volumes of blood and soft tissue, respectively, for Ca^{47} or, of course, for calcium since the contents of the compartments would have the same specific activities.

$$Ca_B{}^{47}/Ca_B = Ca_{ST}{}^{47}/Ca_{ST}$$

The integrator representative of Ca^{47} in the feces in Fig. 2 is also absent in Fig. 1 for technological operation of this particular computer system. The Ca^{47} eliminated from the blood by this route is only

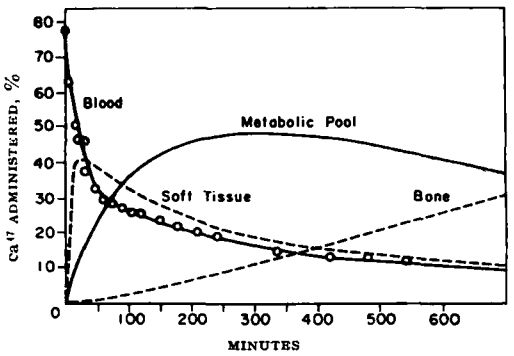

Fig. 5.—Ca^{47} as per cent of intravenously administered dose in the various depots of Dog 1, recovery phase *C vs.* time up to 700 minutes on the basis of a good fit to the plotted blood serum data. The other plots are the "read-out" of the analog computer in accordance with the program of Fig. 2.

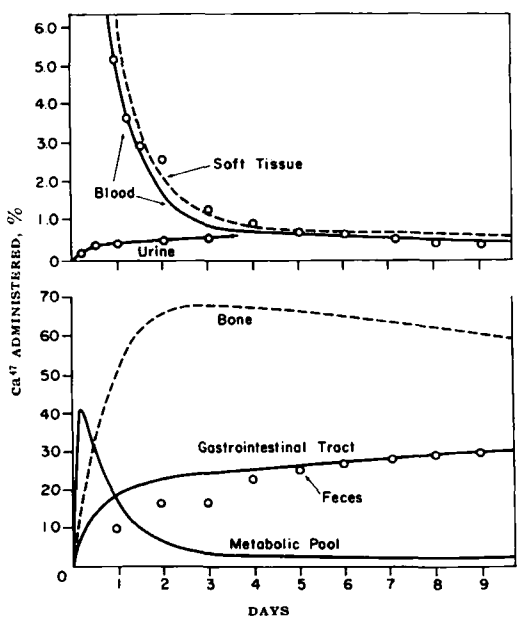

Fig. 6.—Ca^{47} as per cent of intravenously administered dose in the various depots of Dog 1, recovery phase *C vs.* time up to 9 days on the basis of a good fit to the plotted blood serum data and the latter values in the feces. The other plots are the "read-out" of the analog computer in accordance with the program of Fig. 1. A portion of the urine is plotted for comparison.

TABLE IV.—RATE CONSTANTS[a] OF *In Vivo* TRANSFERENCES OF Ca^{47} IN THE DOG AS DERIVED FROM THE FITTING OF BLOOD SERUM AND FECAL Ca^{47} DATA *vs.* TIME BY THE ANALOG COMPUTER

Dog	Study[b]	$10^{2}k_{B\to ST}$	$10^{2}k_{ST\to B}$	$10^{4}k_{B\to MP}$	$10^{5}k_{MP\to B}$	$10^{6}k_{MP\to PB}$	$10^{7}k_{PB\to MP}$	$10^{5}k_{B\to GI}$
1	A	2.26	1.50	3.52	4.77	2.18	1.98	0.67
	B	1.11	0.89	1.74	5.09	2.27	1.19	1.73
	C	1.65	1.45	1.94	4.73	1.78	1.11	1.01
2	A	1.55	1.15	3.33	4.18	2.11	1.48	0.76
	B	1.52	1.36	0.92	2.52	2.47	2.18	1.27
	C	2.11	1.37	1.63	2.92	1.17	0.91	1.33
3	A	2.15	1.21	2.63	2.22	1.67	1.92	0.91
	B	1.10	0.97	1.52	2.68	1.39	0.87	1.75
	C	3.14	2.64	2.71	5.00	1.84	1.32	1.09
4	A	2.13	1.24	3.38	3.34	2.06	1.67	0.95
	B	1.67	0.95	1.88	3.35	1.67	1.11	1.91
	C	1.35	0.87	1.50	2.66	2.00	0.98	0.93

[a] k_i in sec.$^{-1}$ (See model in Fig. 1.) [b] Study A was the control study; Study B was during 2 mg./Kg./day steroid administration; the recovery Study C was conducted after cessation of steroid administration.

experimentally manifested in fecal elimination. However, the effect on Ca^{47} levels in the blood resulting from the transfer of Ca^{47} into the gastrointestinal fluid most certainly occurs relatively quick in comparison to the lag in the determination of Ca^{47} in the feces.

Also apparent first-order fecal eliminations of Ca^{47} would obviously serve as a gross oversimplification of the complicated processes of absorption into the gastrointestinal tract, including binding with fecal matter and subsequent mechanical displacement down the colon.

The computer used was a modified Heath analog computer and the recorders were Mosely model 4.

Fitting the Model to the Analog Computer.—The blood Ca^{47} for the first 700 minutes (real time, Figs. 3 and 5) where computer drift is not operative is fitted by the computer program of Fig. 2 with the processes from the gastrointestinal tract to the feces omitted. The program was revised for the 10-day scale (Figs. 4 and 6) in accordance with Fig. 1. No discontinuities in the fit were observed.

The equilibration constant K and the rate constants $k_{B\to ST}$ and $k_{ST\to B}$ were derived from the original polyexponential fit. The k_1 of the first exponential (Table I) was considered equal to the sum of the forward and reverse rate constants of the model

$$k_1 = k_{B\to ST} + k_{ST\to B} \qquad \text{(Eq. 3)}$$

The apparent soft tissue (ST) compartment size A_1 is also derived from the first exponential of the polyexponential fit. Thus the equilibrium constant, K, for Ca^{47} distributed between the soft tissue and the blood would be

$$K = A_1/(\Sigma A_i - A_1) = k_{B\to ST}/k_{ST\to B} = C_{ST}/C_B \qquad \text{(Eq. 4)}$$

where ΣA_i is the extrapolated value of the Ca_B^{47} content of the blood at zero time (of intravenous administration).

From the data ΣA_i, A_i and k (given in Table I and Eqs. 3 and 4), $k_{B\to ST}$ and $k_{ST\to B}$ can be derived. These values are given in Table IV and were used in fitting the first 700 minutes of Ca_B^{47} data (Figs. 3 and 5). They were also used to calculate the ratio of the two capacitors (Eq. 4), C_{ST} and C_B, representing blood and soft tissue compartmental size, respectively, as used in the program of Fig. 1 and in the curve fitting of Figs. 4 and 6.

After the insertion of the appropriate values for the capacitors and resistors representative of $k_{B\to ST}$ and $k_{ST\to B}$, the capacitors and resistors associated with the rate constants $k_{B\to MP}$ and $k_{MP\to B}$ representative of the blood $\rightleftarrows$ metabolic pool equilibrium were then adjusted until a reasonable fit for the data over the first 100–150 minutes (Figs. 3 and 5) were obtained. A condition for this initial fit was that the constant, k_2, for the second exponential of the polyexponential fit, $\Sigma_i A_i e^{-k_i t}$ can be defined

$$k_2 = k_{B\to MP} + k_{MP\to B} \qquad \text{(Eq. 5)}$$

The 10-day scale (Figs. 4 and 6) was mounted on the recorder and the machine-time/real-time ratio adjusted accordingly. The extrapolated value of Ca_B^{47} at zero time, $(Ca_B^{47})_0$, *i.e.*, the per cent of total administered Ca^{47} per ml. of serum at the time of intravenous administration, was considered equivalent to 100% of the administered radioisotope. Thus, 100 $Ca_B^{47}/(Ca_B^{47})_0$ could be considered equivalent to the per cent of Ca^{47} intravenously administered that was in the blood at any time. The Ca^{47} fecal content was plotted cumulatively as ΣP_i on this same ordinate as per cent of Ca^{47} administered.

The program of Fig. 1 was then used to obtain appropriate $k_{B\to GI}$ and $k_{MP\to PB}$ values by adjustment of resistors and capacitors. The $k_{B\to GI}$ value was initially chosen so as to fit the Ca_B^{47} data and to be consistent with the P_∞ asymptote approached by the cumulative ΣP_i values of the feces at *ca.* 4–5 days, on the assumption of a first-order fecal elimination of Ca^{47}. The $k_{MP\to PB}$ value was concomitantly chosen to fit the Ca_B^{47} data and account for the Ca^{47} not in the blood or feces.

Subsequently, a $k_{PB\to MP}$ value was chosen to fit the positive linear deviation of Ca^{47} fecal excretion, ΣP_i, from the apparent asymptote, P_∞. Minor readjustments were made to the $k_{B\to GI}$ and $k_{MP\to PB}$ constants when necessary.

The 700-minute (Figs. 3 and 5) and 10-day (Figs. 4 and 6) scales were alternately reinserted in the recorder and since all of these rate constants were interacting, fine adjustments in their values were made to assure consistency with the Ca_B^{47} data of the blood, the amounts excreted in the feces, gastrointestinal tract, GI, (in excess of 5 days), and the amounts of Ca^{47} that would have to be in the permanent bone, PB, and metabolic pool, MP, to account for the stoichiometry.

For ease of fitting, the several per cents of the

administered Ca^{47} that appeared in the urine were ignored (Figs. 4 and 6) since their overall contribution was not of great significance.

The rate constants so determined are given in Table IV. They were calculated from the relation

$$k(\text{sec.}^{-1}) = P/RC \times (\text{machine time})/(\text{real time}) \quad \text{(Eq. 6)}$$

where P is the potentiometer setting, R is the resistance in megohms, and C is the capacitance in microfarads.

The output of each compartment was graphed on the recorder as a function of time after the intravenous administration of Ca^{47}. Typical plots are given in Figs. 3–6.

RESULTS

Empirical Equations Fitted to the Ca^{47} Content of the Blood.—The parameters of the four-factor linear homogeneous equation of exponentials, $\Sigma_i A_i e^{-k_i t}$, given in Eq. 2 and representative of the disappearance of Ca^{47} from the blood of the four dogs during a control phase, A, a steroid regimen, B, and a recovery phase, C, are given in Table I.

As previously noted (1), no specific or consistent patterns appear in the k_i values that can be ascribed to the effect of steroid regimen or steroid recovery.

The major difference within dogs appears in the magnitude of the A_i values, the total amount of the Ca^{47} transferred from the blood to the four hypothetical compartments. This is most readily demonstrated in the plots of $Ca_B{}^{47}$ (as per cent of the total administered dose per ml. of serum) in the blood *versus* time for the four dogs. In general, the curves were similar but displaced along the ordinate. There is a significantly higher concentration of $Ca_B{}^{47}$ in the blood for the steroid phase than for the control phase in all cases.

The $Ca_B{}^{47}$, before the A_4 value of the fourth exponential becomes the determining factor (<750 minutes) and after the fast decrease in $Ca_B{}^{47}$ due to the first exponential (>30 minutes) has been expended, is lower in the recovery phase than in the steroid phase. However, the $Ca_B{}^{47}$ did not reach the lower levels attained with the dog as his own control nor was it as significantly different from the $Ca_B{}^{47}$ values for the steroid phase. The curves of $Ca_B{}^{47}$ against time are similar to those given in the figures of the prior paper in this series (1, 2). Figure 1a of the latter reference (2) is representative of the data.

It has been previously shown (1) that the A_1 (in three out of four dogs), A_2 (in four out of four dogs), and A_3 and A_4 (in three out of three dogs) were all lower for the control period than for the steroid regimen studies. With minor exceptions, these A_1, A_2, and A_3 values decreased for the recovery periods. There were no highly significant differences between the A_4 values of steroid phases and the subsequent recovery A_4 values.

The present study confirmed a good part of these observations (Table I). The major differences in the A_i values that account for the displacement of the Ca^{47} in blood serum curves are in the A_3 and A_4 values. In four dogs out of four the steroid regimen phases have higher A_3 and A_4 values than the control.

The slight decrease in $Ca_B{}^{47}$ values (30 minutes > t < 750 minutes) from steroid to recovery phase may be accounted for by the lessened value of A_3 (for three out of four dogs) (Table I). There were no significant differences in A_4 values between steroid and recovery phase.

The A_1 and A_2 data did not permit conclusions regarding differences among phases of treatments.

Empirical Equations for Ca^{47} Content of the Feces and Urine.—The appearance of Ca^{47} in the feces and urine has been represented by apparent first-order expressions (1)

$$\Sigma P_i = \text{Accumulated } Ca^{47} \text{ in feces (or urine)} = P_\infty(1 - e^{-kt}) \quad \text{(Eq. 7)}$$

where P_∞ is the total per cent of the total dose that should appear in the feces (or urine) over the entire period on the postulate of first-order kinetics. The two pertinent parameters are P_∞ and k, and are given in Table II for all dogs and phases as best fitted by the analog computer (1). Again, as in the previous study (1), the amount of Ca^{47}, *i.e.*, P_∞ appearing in the urine and feces by an assumption of first-order kinetics is greatly enhanced by a steroid regimen, Run B over Run A. (See Fig. 7 for Dog 3.)

In fact, the increase in fecal elimination for these studies on steroid regimen is even more pronounced than previously (1), primarily due to the lower P_∞ values for the dogs in the control phases of the first studies. The P_∞ values for dogs of similar age during the steroid regimen were similar, *ca.* 30% of the total Ca^{47} administered.

The "rebound" effect or decrease in P_∞ for the recovery phase (Table II) was definitely present (Fig. 7) but not as pronounced in the present studies since the P_∞ values for the control phases were so much lower. However, P_∞'s for the recovery phases were of the same magnitude in both sets of studies.

The apparent rate constant, k, for the fecal and urinary elimination among runs for particular dogs did not significantly correlate with the conditions of the run.

As was also demonstrated in other papers of this series (1, 2), the fecal (and urinary) elimination of Ca^{47} deviates from first order by an amount of radioisotope excreted in excess of an expected asymptote,

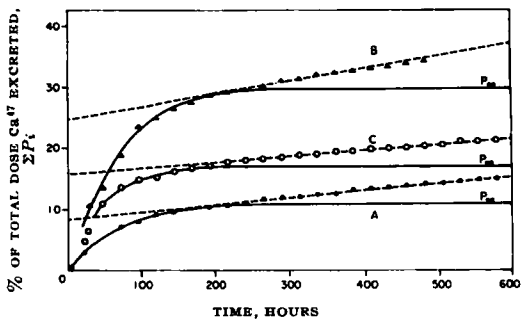

Fig. 7.—Typical example of cumulative fecal excretion of Ca^{47} with time over 600 hours. The data is for Dog 3; A is control study, B is steroid study, and C is recovery study. The solid curves represent the pseudo first-order fecal excretion with time, $\Sigma P_i = P_\infty(1 - e^{-kt})$. The dashed lines represent the apparent linear deviation from the first-order asymptote P_∞, *i.e.*, $\Sigma P_i = mt + b$.

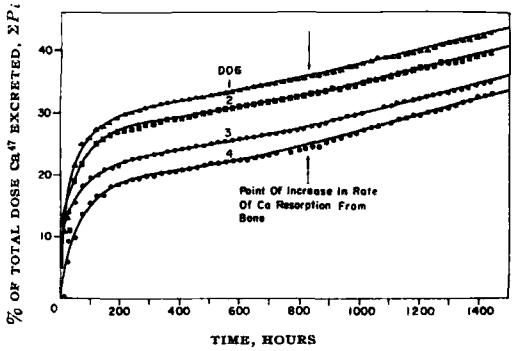

Fig. 8.—Cumulative fecal excretion of Ca^{47} with time for the recovery phases of the dogs over 1500 hours. For reasons of clarity, the data are plotted as Y for Dog 1, Y-4.8% for Dogs 2 and 3, and Y-12% for Dog 4.

i.e., P_∞. This deviation can be approximated by a straight line of slight slope

$$\Sigma P_i = mt + b, \quad t > 200 \text{ hr.} \qquad \text{(Eq. 8)}$$

The slope, m, is the per cent of the total Ca^{47} dose excreted per hour and b is the intercept. The m and b values are given in Table II for both urinary and fecal elimination. Typical plots of this linear deviation are given for the fecal excretion of Dog 3 in Fig. 7.

Since the m values may serve as estimates of the possible rates of reabsorption of Ca^{47} from bone, these values should be adjusted to account for any bias introduced by greater amounts of radioisotope in bone. A reasonable adjustment can be made by dividing m by per cent of the dose of administered Ca^{47} in the bone. This per cent (of the total dose) of Ca^{47} in the bone can be estimated from the difference between 100% and the sum of the apparent first-order asymptotes of Ca^{47} excretion in urine and feces reached at *ca.* 200 hours. Thus

$$R = m/[100 - P_\infty(\text{urine}) - P_\infty(\text{feces})] \qquad \text{(Eq. 9)}$$

where the apparent resorption rate R is the Ca^{47} available for excretion into the feces per unit of Ca^{47} in the bone. This value may be a satisfactory measure of the calcium resorption from bone.

The R data of Table II show that the Ca^{47} reabsorption rate is almost double that of control for all cases of steroid administration and relapses on recovery to at least the rate of the control period and probably less. This phenomenon is also mirrored by the urine data.

Subsequent to this linear deviation from the expected first-order asymptote of Ca^{47} excretion, a further increase in bone resorption rate was observed when the fecal collections were carried out in excess of 700 hours during the recovery phases. The plots are given in Fig. 8.

This new enhancement of resorption rate can be characterized by the linear expression

$$\Sigma P_i = m'(t - t') + b' \qquad \text{(Eq. 10)}$$

where m' represents the new rate and b' is the value of Ca^{47} accumulated in the feces at time $t = t'$, *ca.* 700–800 hours, which represents the time of transition from the old to the new resorption rate.

Again, the m' for the various dogs can be adjusted for Ca^{47} content in the bone, dividing m' by the per cent of total Ca^{47} dose in the bone. The value $100 - b'$ is considered a good estimate of this latter value. Thus

$$R' = m'/(100 - b') \qquad \text{(Eq. 11)}$$

where the R' is the estimated second linear rate of Ca^{47} available for excretion into the feces per unit of Ca^{47} in the bone. This value may be a satisfactory measure of the calcium resorption from bone 700 hours after Ca^{47} administration and during the recovery phase. The m', b', t', and R' values are given in Table III.

Extended studies of fecal elimination during the recovery phase showed that the estimated rates of Ca^{47} resorption subsequently increased by *ca.* 20–40% over the rates of resorption during the 200–700 hour period post-Ca^{47} injection.

Rates of Distribution of Intravenously Administered Ca^{47} as Determined by the Analog Computer.—The apparent first-order rate constants for the compartmental model in Fig. 1 are listed in Table IV. There are no consistent effects of steroid administration and subsequent recovery on the apparent first-order rate constants for the transfer of Ca^{47} from the blood, B, to the soft tissue, ST, *i.e.*, $k_{B\to ST}$ and $k_{ST\to B}$. The relative apparent volume (14) of the soft tissue, V_{ST}, available for Ca^{47} to the blood serum apparent volume, V_B

$$V_{ST}/V_B = k_{B\to ST}/k_{ST\to B} = Ca_{ST}/Ca_B \qquad \text{(Eq. 12)}$$

is of the order 1.2–1.7 (average 1.4) among dogs. This may also be interpreted as the ratios of the calcium contents of both compartments. (See *Appendix.*)

A satisfactory estimate of half the time it would take to reach equilibrium between the blood and soft tissue is 4 minutes, a value which is similar to that previously determined (1).

A definitive decrease in the rate constant assigned to the transfer of Ca^{47} from the blood to the metabolic pool, $k_{B\to MP}$, is observed for steroid over the control period. In three out of four dogs an increase in the rate of return of Ca^{47} from the metabolic pool to the blood, $k_{MP\to B}$, was observed after cessation of steroid.

The ratio of these rates, $k_{B\to MP}/k_{MP\to B}$, may be more indicative of the steroid effect on Ca^{47} distribution. In the control phase the ratio (average 9.4) is twice the value of the ratio while steroid is administered (average 4.6) which is almost equivalent to the value of the ratio during the recovery phase (average 5.2).

An alternate method of appraising this data is by examining the apparent relative volumes or calcium contents of the compartments (14–16). From this perspective, the capacity of the metabolic pool for Ca^{47} and calcium with respect to blood serum may be considered as halved on steroid administration. This relative decrease in the capacity of the metabolic pool remained after cessation of steroid in these studies.

With the model used the major source of the differences in $Ca_B{}^{47}$ values with time within a dog can be attributed to the blood-metabolic pool equilibrium and the blood-gastrointestinal tract transfer, $k_{B\to GI}$.

The values assigned this latter rate constant almost double during steroid phase with respect to the control. On recovery from steroid, they decidedly tend to revert to the control levels.

The introduction of a $k_{PB \rightarrow MP}$ rate constant to account for the return of Ca^{47} from the bone to the metabolic pool is obviously an oversimplification of a complex process. It is significant, however, that the apparent linear increase in Ca^{47} excretion after 5 days (Figs. 4 and 6) can be accounted for by this assumption that Ca^{47} is resorbed from the bone.

DISCUSSION

Most Significant Steroid Effects.—The results of these detailed studies of intravenously administered Ca^{47} in young Beagles support and extend those previously published (1, 2). The most significant effects of steroid radiocalcium metabolism include the increased amounts (more than twice control) of Ca^{47} accumulated in the feces (P_∞ in the Table II) and the increased rate of apparent resorption (*ca.* twice control) of Ca^{47} from a "deep" compartment that may be assigned to bone (R in Table II). Both of these effects are decidedly reversed on the cessation of steroid administration.

Explanation and Correlation of Curve-Fitting.—The fitting of the data by the analog computer (Fig. 1) permits an explanation of these phenomena. It also permits a correlation of the parameters of the model with the sum of exponentials, Eq. 2, fitted to the Ca_B^{47} decrease with time.

The first exponential, Table I, and the $k_{B \rightarrow ST}$ and $k_{ST \rightarrow B}$ (Table IV) values are synonomous in their significance, the presumed fast transfer of Ca^{47} into extravascular spaces and soft tissues. The second exponential (Table I), $A_2e^{-k_2t}$, is largely representative of a slower transfer to the compartment represented as the metabolic pool (Figs. 1, 3, 5). This compartment could be assigned to the reasonably reversible chelation and/or complexation of calcium with bone or matrix.

The analog computer fitting of this model, the ratio $k_{B \rightarrow MP}/k_{MP \rightarrow B} = Ca_{MP}/Ca_B$ (Table IV), which decreases with respect to control on steroid administration, may explain the apparent increase in Ca^{47} blood levels during steroid, *i.e.*, the relative increase in A_3 and A_4 values (Table I). The relative constancy of this ratio between steroid and recovery studies would also explain the fact that the polyexponential A_3 and A_4 values do not appreciably differ between steroid and recovery (Table I).

The model fitting shows that the inhibition of transfer of Ca^{47} from the blood to the metabolic pool, $k_{B \rightarrow MP}$, by a presently unknown mechanism of steroid action, is a plausible explanation for steroid induced osteoporosis. If this metabolic pool represents the sites from which the radioisotope is incorporated into a deep compartment such as bone by the assigned rate constant, $k_{MP \rightarrow PB}$ (Table IV), there does not have to be significant effects of steroid on this latter transfer. This was indicated by the lack of a significant variation of $k_{MP \rightarrow PB}$ with treatment within the dogs. The decrease of Ca^{47} in bone on steroid-induced osteoporosis is a ready explanation on the premise that the amount of calcium in the metabolic compartment necessary for bone incorporation is lessened.

This hypothesis would be consistent with the postulates that an effect of the adrenal steroids is to inhibit the synthesis of protein matrix (17, 18). Thus, less sites would be available for a comparatively reversible binding of calcium with bone precursor as represented by the values for the ratio, $k_{B \rightarrow MP}/k_{MP \rightarrow B}$, as modified by steroid (Table IV).

The recovery period studies again demonstrate reversal of steroid effects in that an increase in the $k_{B \rightarrow MP}$ value is observed on cessation of steroid.

The model used and as fitted (Fig. 1) showed a marked increase with steroid in the apparent first-order rate constant, $k_{B \rightarrow GI}$, for the transfer of Ca^{47} across the intestinal wall or by biliary or gastric secretion as crudely estimated from the fecal excretion data. As has been stated previously (2), this is representative of the excretion of endogenous calcium and is explained by a steroid mechanism that would increase the rate of calcium transfer from the blood to the intestine. The possibility of decreased calcium binding by protein, thereby increasing the amounts of diffusible calcium has already been cited (2, 19) and is consistent with this data. An alternative explanation (2) is the inhibition of calcium return *via* gastrointestinal absorption which is also consistent with recent reports (20).

The analog computer plottings (Figs. 4 and 6) even with an oversimplified first-order process for Ca^{47} transfer from bone to metabolic pool, show that the positive deviation from first-order fecal and urinary eliminations (Table II, Figs. 7 and 8) can be accounted for by a resorption of radioisotope from bone, at least for the data up to 10 days (Figs. 4 and 6).

Limitations of Studies to Date.—The great numbers of fecal, urinary, and blood serum collections necessary to do studies of this kind decidedly limit the number of animals that can be followed experimentally. The major arguments for these experimental designs (within the limitations of the amount of data that can be practicably obtained) are that each dog can be used as its own control and that the relatively short half-life of Ca^{47} permits repetitive radioisotope labeling without interference from the radioisotope administered in the previous study.

The dogs used in this study were in a maximum growth or maturation period and, undoubtedly, hormonal changes occurred in this interval which may have affected their calcium metabolism. It is known that the retention of radioisotopes decreases with increase in age of the dog (21). However, it is felt that such a change would be unidirectional and that when effects of steroid are reversed on recovery, the validity of these effects may be given additional weight. It has been shown that the nature of these effects is similar in the adult dog (1). It is also argued that less variability exists among "litter-mates" during their youth.

Detailed studies should be made with varying steroid dosages to evaluate dose-response relations. Cold calcium assays should be determined simultaneously with Ca^{47} concentrations to correlate changes in specific activity with the influences of adrenal steroid administration and to permit the calculation of exchange rates, r, between compartments rather than the fractional exchange rates, $r/Ca_i = k_i$, which merely permit estimates of relative compartment sizes or the relative calcium content of exchanging compartments. (See *Appendix*.)

The interesting observation of a significant and sudden increase in the apparent resorption rate of Ca^{47} from bone on recovery from steroid should be further investigated. The reproducibility of these Ca^{47} methods without steroid, *i.e.*, the maturation effect on radiocalcium metabolism should be evaluated. Such studies and their results will be reported in a future publication.

Advantages of the Application of the Analog Computer.—The analog computer is an extraordinary tool for the elucidation of the mechanisms and rates of transformations and compartmental exchanges of drugs in the various organs of the body. It permits the establishment of models for gastrointestinal absorption into the blood, diffusion into tissues, incorporation into organs, metabolic pathways, and elimination into the urine and feces. Its use permits the investigation and quantification of distribution and excretion processes for which the application of methods of analytical mathematics is most approximate and difficult.

The philosophy of analog computer programming and application to be consistent with experimental assay, blood level, urinary, and fecal data is based on the use of minimum postulates consistent with physiological reality. The validity of "steady state" approximations and "apparent metabolic half-lives" with respect to the more exact models programmed in the analog computer can be evaluated as is shown in the *Appendix*.

Comparisons of empirical curve fitting and model making by the analog computer were made with respect to Ca^{47} dynamics.

Of course, the digital computer could also be used since numerical methods could approximate the solutions of differential equations established for the appropriate mathematical models. However, the analog computer has the advantages of simpler and more flexible programming for various models. It can generate a series of curves which can be observed by the operator as consistent or inconsistent with the forms and magnitudes of plotted data.

APPENDIX

The total Ca_T^{47} or 100% of the administered dose can be considered as distributed among the various compartments as blood, Ca_B^{47}, soft tissue, Ca_{ST}^{47}, metabolic pool, Ca_{MP}^{47}, gastrointestinal tract, Ca_{GI}^{47}, and bone, Ca_{PB}^{47}, in concurrence with the model of Fig. 1, as

$$100\% = Ca_T^{47} = Ca_B^{47} + Ca_{GI}^{47} + Ca_{ST}^{47} + Ca_{MP}^{47} + Ca_{PB}^{47} \quad \text{(Eq. 13)}$$

where Ca_{GI}^{47} also includes the cumulative amount in the feces and where the negligible amounts in the urine can be ignored. (See Figs. 4 and 6.)

The rates of transfer of Ca^{47} among these compartments before resorption from bone becomes significant can be formulated as

$$d\,Ca_B^{47}/dt = -[k_{B\to GI} + k_{B\to ST} + k_{B\to MP}]\,Ca_B^{47} + k_{ST\to B}Ca_{ST}^{47} + k_{MP\to B}Ca_{MP}^{47} \quad \text{(Eq. 14)}$$

$$d\,Ca_{GI}^{47}/dt = k_{B\to GI}Ca_B^{47} \quad \text{(Eq. 15)}$$

$$d\,Ca_{ST}^{47}/dt = k_{B\to ST}Ca_B^{47} - k_{ST\to B}Ca_{ST}^{47} \quad \text{(Eq. 16)}$$

$$d\,Ca_{MP}^{47}/dt = k_{B\to MP}Ca_B^{47} - (k_{MP\to B} + k_{MP\to PB})Ca_{MP}^{47} \quad \text{(Eq. 17)}$$

$$d\,Ca_{PB}^{47}/dt = k_{MP\to PB}Ca_{MP}^{47} \quad \text{(Eq. 18)}$$

Alternately, these apparent rate constants can be interpreted as fractional turnovers or the fraction of compartmental calcium exchanged in a unit of time as they represent the quotient of an amount of calcium transferred from a compartment in a unit of time, r, and the total amount of calcium, Ca_i, in that compartment, r/Ca_i (14, 15). For example, Eq. 16 can be formulated as the rate of radioisotope transfer proportional to the specific activities of the blood and soft tissue compartments

$$d\,Ca_{ST}^{47}/dt = r[S_B - S_{ST}] = (r/Ca_B)Ca_B^{47} - (r/Ca_{ST})Ca_{ST}^{47} \quad \text{(Eq. 19)}$$

The $S_B = Ca_B^{47}/Ca_B$, $S_T = Ca_{ST}^{47}/Ca_{ST}$ represent the specific radioactivities in the blood and soft tissue compartments, respectively, and Ca_B and Ca_{ST} are the respective total calcium contents of these compartments, so that $k_{B\to ST} = r/Ca_B$ and $k_{ST\to B} = r/Ca_{ST}$. It also follows that $k_{B\to ST}/k_{ST\to B} = Ca_{ST}/Ca_B$ is the ratio of the calcium in the soft tissue to the amount in the blood. It may also be interpreted as the ratio of the apparent effective volumes of soft tissue and blood with respect to calcium if the concentration per unit volume is considered to be the same in the equilibrated compartments.

Inspection of Figs. 4 and 6 indicates that at 1–3 days after the intravenous administration of Ca^{47}, pseudo or quasi steady state conditions can be assumed for the model given in Fig. 1. This assumption implies that the rates of Ca^{47} transfer between blood and soft tissue and blood and metabolic pool are no longer rate determining in the decrease of Ca_B^{47}, and the amounts of radioisotope in these various compartments are proportionately related, as

$$Ca_{MP}^{47} = (k_{B\to MP}/k_{MP\to B})Ca_B^{47} = (Ca_{MP}/Ca_B)Ca_B^{47} \quad \text{(Eq. 20)}$$

$$Ca_{ST}^{47} = (k_{B\to ST}/k_{ST\to B})Ca_B^{47} = (Ca_{ST}/Ca_B)Ca_B^{47} \quad \text{(Eq. 21)}$$

These equations also state that the specific radioactivities, S, in all three compartments are the same in the pseudo or quasi steady state as from Eqs. 20 and 21

$$S = Ca_B^{47}/Ca_B = Ca_{MP}^{47}/Ca_{MP} = Ca_{ST}^{47}/Ca_{ST} \quad \text{(Eq. 22)}$$

The rates of increase of radioisotope in the gastrointestinal tract and bone (Eqs. 15 and 18) may be formulated as equal to the rates of decrease in metabolic pool, soft tissue, and blood (Eqs. 14, 16, and 17) before any return of Ca^{47} from bone to metabolic pool significantly contributes

$$d\,Ca_{GI}^{47}/dt + d\,Ca_{PB}^{47}/dt = -d[Ca_{MP}^{47} + Ca_{ST}^{47} + Ca_B^{47}]/dt = k_{B\to GI}Ca_B^{47} + k_{MP\to PB}Ca_{PB}^{47} \quad \text{(Eq. 23)}$$

Substitution of Eqs. 20 and 21 into Eq. 23 and rearranging gives

$$-d\,Ca_B^{47}/dt = \left[\frac{k_{B\to GI} + k_{MP\to PB}\,k_{B\to MP}/k_{MP\to B}}{1 + k_{B\to MP}/k_{MP\to B} + k_{B\to ST}/k_{ST\to B}}\right] Ca_B^{47} = k_4 Ca_B^{47} \quad \text{(Eq. 24)}$$

The estimated apparent first-order rate constant for the pseudo or quasi steady state elimination of radioisotope from the blood could also be expressed as

$$k_4 = \left[\frac{r_{B,\,GI} + r_{MP,\,PB}}{Ca_B + Ca_{MP} + Ca_{ST}}\right] \quad \text{(Eq. 25)}$$

by substituting the appropriate r_i/Ca_i for the k_i of Eq. 24 and simplifying the result. The r_i values are the calcium exchange rates between the two compartments given in the subscripts of the k_i; the Ca_i values are the total calcium in the compartments identified by the subscript.

It can also be shown (16, 22) from Eqs. 13, 20–22 that on pseudo steady state conditions

$$d\,Ca_{GI}{}^{47}/dt + d\,Ca_{PB}{}^{47}/dt = k_4[Ca_T{}^{47} - Ca_{GI}{}^{47} - Ca_{PB}{}^{47}] \quad \text{(Eq. 26)}$$

This estimated apparent first-order rate constant, k_4, for the pseudo steady state elimination of radioisotope from the blood could also be expressed in terms of apparent compartmental volumes (16, 22, 23).

The term, k_4 (as used in Eqs. 24–26) should be analogous to the k_4 in Table I, the apparent decay constant for the fourth exponential of the polyexponential fit of the $Ca_B{}^{47}$ *versus* time data. Substitution of the k_i values given in Table IV for the model of Fig. 1 will give calculated k_4 values from Eq. 23 of 3×10^{-6} sec.$^{-1}$, whereas the k_4 values of 5×10^{-6} sec.$^{-1}$ are listed in Table I.

The model used in the programming of the computer was necessarily a simplification of the physiological reality. The estimates of $k_{B\to GI}$ representative of apparent rates of radioisotope transfer from the blood to the gastrointestinal tract were actually determined by analog computer fitting to the cumulative fecal Ca^{47} at 5–10 days since no actual data were available for the amount in the intestine. The true value of $k_{B\to GI}$ is undoubtedly greater than the first approximation from fecal data since intestinal capacity for Ca^{47}, possible fecal and mucosal binding of radioisotope, and lag in defecation would contribute to an underestimation of the rate of removal of $Ca_B{}^{47}$ from the blood to the intestine.

An increased value of $k_{B\to GI}$ in Eq. 23 would estimate a k_4 value more consistent with the listed values of Table I. Further, the model and program of Fig. 1 ignore the probable equilibration of Ca^{47} in the intestinal tract with that of the blood as was indicated in the more complex model given in Fig. 2.

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Psychopharmacological Studies of Some 1-(Chlorophenyl)-2-aminopropanes I

Effects on Appetitive-Controlled Behavior

By JOHN E. OWEN, JR.

A series of 1-phenyl-2-aminopropanes with chloro substitutions on the 2,4, 3,4, and 4 positions on the phenyl ring and their optical isomers were compared with *dl*- and *d*-amphetamine and methamphetamine on a fixed-ratio (FR) procedure. The subjects were rats that had been trained to press a lever 30 times for 0.25-ml. reinforcement of sweetened condensed milk. Each session consisted of 40 reinforcements. With unsubstituted amphetamines, rats showed a slight reduction in response rates with long pauses following reinforcement. This effect was similar to the effect seen with onset of normal satiation. After chloro compounds, the rats produced irregular and slowed response rates with frequent short pauses between and following reinforcement. The minimum effective doses of all compounds used in this study ranged from 1–3.2 mg./Kg. Except for *d*-1-(4-chlorophenyl)-2-aminopropane, the *l*-isomers of chloro-substituted compounds were more active suppressants of FR behavior than the *dl* or *d* compounds.

COMPOUNDS THAT stimulate the central nervous system and also have appetite-suppressing activity, such as amphetamine and methamphetamine, have be enreported to alter performance of trained experimental animals working for food reinforcement on fixed-ratio (FR) schedules (1–3). Specifically, *dl*-, *d*-, and *l*-amphetamine and methamphetamine (4) changed FR behavior in rats by slowing the response rate, increasing the frequency and duration of pauses between and following reinforcements, and inducing temporary cessation of responding following drug administration. Some chloro substituted 1-phenyl-2-aminopropanes in a series of phenylalkylamines studied for central-nervous-system activity by Rathbun (5) exhibited appetite suppression with minimum stimulation. Rathbun estimated the appetite suppressing ED_{50}'s of these compounds in carefully controlled studies on the food intake of hungry rats under the influence of the drugs. Four of these chloro-substituted compounds and their optical isomers were selected for comparison with *dl*- and *d*-amphetamine and methamphetamine on an FR schedule for food reinforcement. The changes in the appetitive controlled behavior of rats after the administration of the drugs is described.

EXPERIMENTAL

Materials.—The compounds and doses used in this investigation as well as the oral ED_{50} doses (5) are listed in Table I. The compounds were dissolved in distilled water and administered subcutaneously.

Method.—Male rats of a Wistar-derived strain, initially weighing 400–450 Gm., were used in this study. With controlled food deprivation their body weights were gradually reduced to 70% of the initial weights. They were trained on an FR-30 schedule in which 30 lever pressing responses were reinforced with 0.25 ml. of a mixture of sweetened condensed milk, water, and homogenized multiple vitamins,[1] in proportions of 1:1:0.01.

The apparatus and procedure have been described in detail elsewhere (4). Briefly, two experimental cages, each 9 × 9 × 7.5 in. were used. Each contained a lever, a modified telephone-type switch (6), a small pilot light over the lever, and a motor-driven dipper for delivery of the reinforcement. The cages were contained in light-proof, sound resistant, ventilated boxes isolated from the control equipment. The procedure, controlled by appropriate electrical relay circuits, was designed to give the rat a reinforcement upon completion of 30 responses. Illumination inside the cage was provided during a session by the small light over the lever. During the 8-second interval that the reinforcement was available to the rat, the light over the lever was extinguished and another small light illuminated the dipper cup.

Each rat was given daily sessions of 40 reinforcements to maintain stable performance. For sessions when drugs were tested, a rat was given ten reinforcements before drug administration as a "warm-up" and to establish that he was working normally that day.

In a previous study (4) the clinically used amphetamines and methamphetamines had been shown to produce maximum suppression of FR responding within 20 to 30 minutes after administration. Therefore, the animal was placed in a small observation cage for 30 minutes following drug administration to allow the drug to be absorbed. The animal was returned to the experimental cage and permitted to work for 40 reinforcements. The compounds were given once to each rat at each dose.

Data.—The data were collected on electrical impulse counters, running-time meters, and cumula-

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[1] Marketed as Homicebrin by Eli Lilly and Co.

TABLE I.—DRUGS AND DOSES USED IN FR SUPPRESSION STUDIES

Drug	Doses, mg./Kg.			Oral ED_{50}, mg./Kg.
dl-Amphetamine Sulfate	1.0	2.0	3.2	...
d-Amphetamine Sulfate	1.0	2.0	3.2	2.65
d-Methamphetamine HCl	1.0	2.0	3.2	...
dl-1-(4-Chlorophenyl)-2-aminopropane HCl	3.2	5.0	8.0	2.05
d-1-(4-Chlorophenyl)-2-aminopropane HCl	1.0	2.0	3.2	1.33
l-1-(4-Chlorophenyl)-2-aminopropane HCl	2.0	3.2	5.0	3.35
dl-1-(2,4-Dichlorophenyl)-2-aminopropane HCl	3.2	5.0	8.0	4.11
d-1-(2,4-Dichlorophenyl)-2-aminopropane HCl	3.2	5.0	8.0	10.00
l-1-(2,4-Dichlorophenyl)-2-aminopropane HCl	2.0	3.2	5.0	2.57
dl-1-(3,4-Dichlorophenyl)-2-aminopropane HCl	2.0	5.0	8.0	2.86
d-1-(3,4-Dichlorophenyl)-2-aminopropane HCl	3.2	5.0	8.0	4.10
l-1-(3,4-Dichlorophenyl)-2-aminopropane HCl	2.0	3.2	5.0	1.81
dl-1-(3,4-Dichlorophenyl)-2-methylaminopropane HCl	3.2	5.0	8.0	4.21
d-1-(3,4-Dichlorophenyl)-2-methylaminopropane HCl	3.2	5.0	8.0	5.69
l-1-(3,4-Dichlorophenyl)-2-methylaminopropane HCl	3.2	5.0	8.0	2.65

tive recorders. Two variables of this appetitive controlled FR behavior were studied: the running response rates, *i.e.*, the sustained constant rate (7), and the total time used to complete each session. The response rates of the individual rats were determined as responses per second. Because this investigation was concerned with the running response rate in contrast to the overall response rate (4, 7, 8), pauses in responding of more than 5 seconds were not included in the response-rate calculations. Since each rat acted as its own control, *i.e.*, normal behavior *versus* behavior with drug, the data from each rat were calculated as ratios of response rates with drug to response rates without drug. Also, ratios of total time to complete a session with drug to mean total time to complete a session without drug were calculated.

The cumulative recorders produced direct recordings of responses related to time. Thus, a visual record of behavior was available at any point of an experimental session. In these records vertical movement of the pen is related to the number of

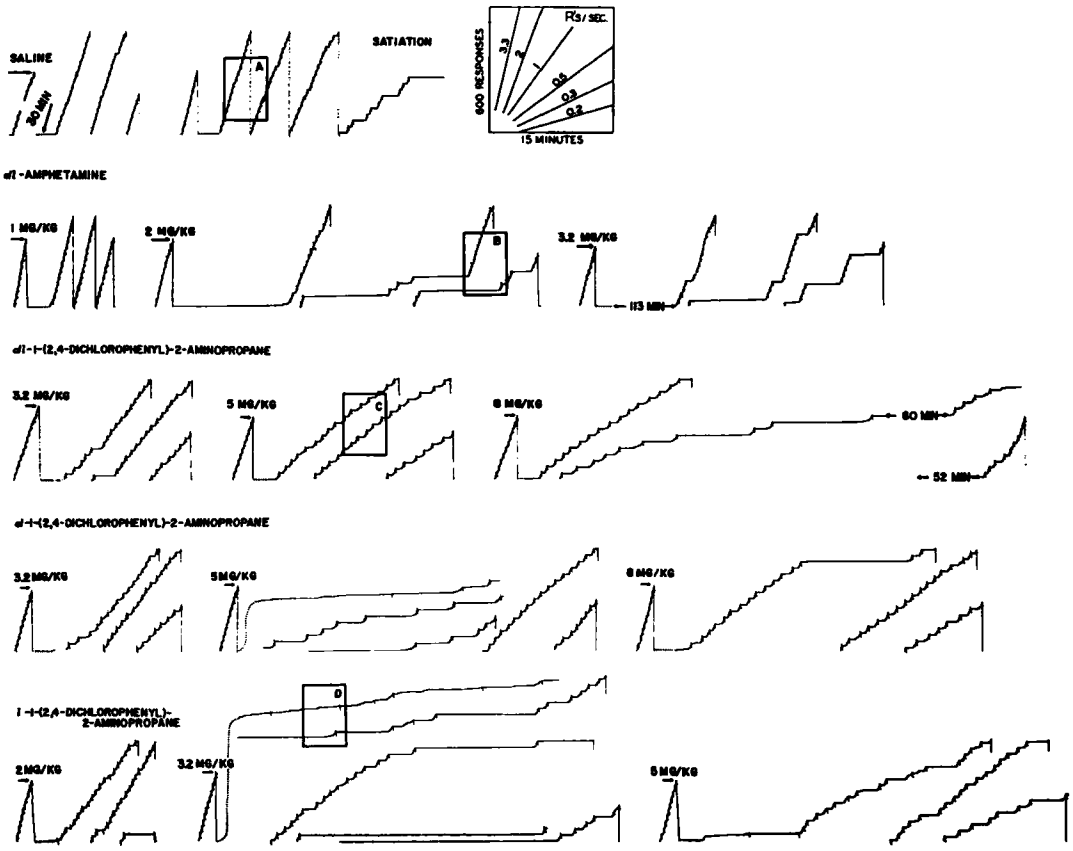

Fig. 1.—Cumulative records of responses of Rat 84 showing patterns of normal behavior and behavior under the influence of the drugs used in this study.

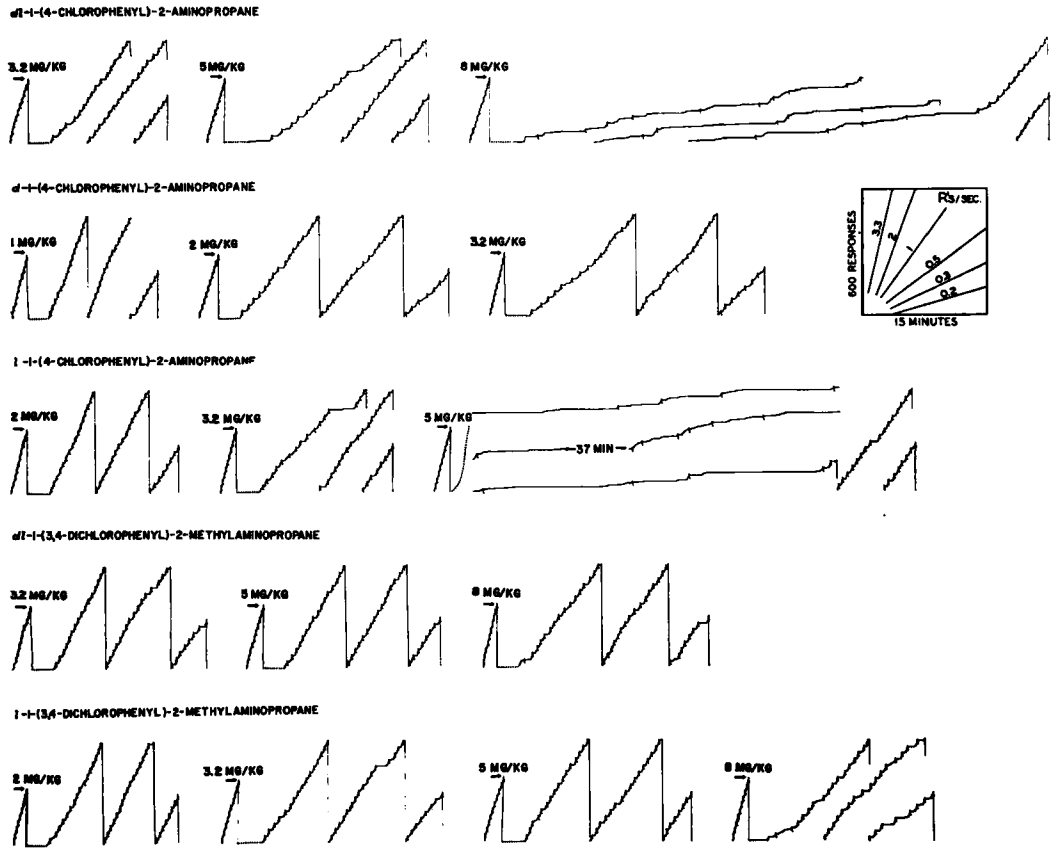

Fig. 2.—Cumulative records of responses of Rat 84 showing patterns of behavior under the influence of drugs used in this study.

responses and horizontal paper movement indicates time. The angle of the line or any segment of the ine is indicative of the individual's response rates. Short vertical marks on the response line show when reinforcements occurred.

RESULTS

The data from this behavioral study of chloro-substituted 1-phenyl-2-aminopropanes, as recorded on the cumulative response recorders, demonstrated that these compounds influence appetitive-controlled FR behavior differently than do *dl*- and *d*-amphetamine and methamphetamine. In general, the deschloro drugs influenced the behavior by causing only a slight depression of response rates with a marked increase in the frequency and duration of the post-reinforcement pauses. At the higher doses some rats also failed to resume responding immediately upon being returned to the cage after the 30-minute period following drug administration.

Figures 1 and 2 present typical cumulative response curves of one rat showing a session with saline; a session during which an unlimited number of reinforcements was made available to the rat (satiation); the effects of *dl*-amphetamine; and the effects of eight of the chloro-substituted compounds. With *dl*-amphetamine there was little variation in the response rate when the rat was working. This rate appeared constant from dose to dose. Shown

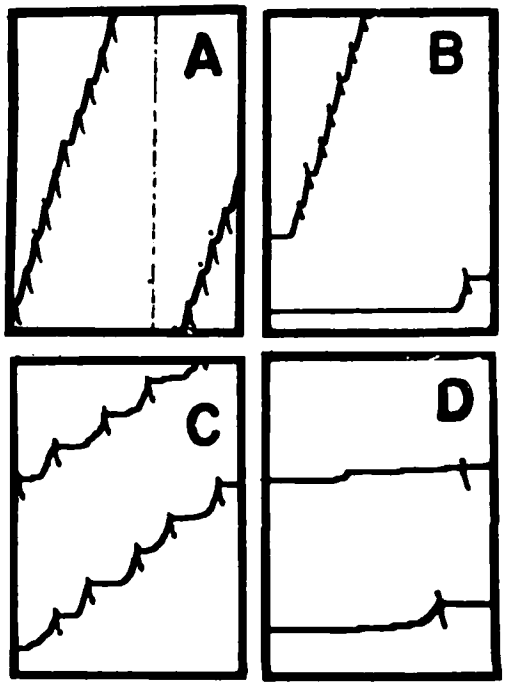

Fig. 3.—Enlarged segments of cumulative record from Fig. 1.

TABLE II.—MEAN RESPONSE-RATE RATIOS

Compound			Rats, No.	Low Dose	Middle Dose	High Dose	Slope, ± S.E.	P
Phenyl Substituent	Amino Substituent	Isomer						
H	H	*dl*	7	0.73	0.64	0.68	−0.025 ± 0.061	0.70
H	H	*d*	7	0.62	0.66	0.66	+0.016 ± 0.039	0.70
H	CH_3	*d*	7	0.84	0.81	0.68	−0.081 ± 0.082	0.45
4-Cl	H	*dl*	5	0.76	0.50	0.32	−0.218 ± 0.079	0.05
4-Cl	H	*d*	5	0.91	0.39	0.38	−0.264 ± 0.037	<0.01
4-Cl	H	*l*	5	0.88	0.56	0.42	−0.229 ± 0.028	<0.01
2,4-diCl	H	*dl*	5	0.74	0.54	0.37	−0.182 ± 0.050	<0.02
2,4-diCl	H	*d*	7	0.68	0.51	0.44	−0.119 ± 0.029	<0.01
2,4-diCl	H	*l*	7	1.03	0.38	0.35	−0.338 ± 0.049	<0.001
3,4-diCl	H	*dl*	5	0.93	0.65	0.55	−0.187 ± 0.081	<0.10
3,4-diCl	H	*d*	5	0.84	0.63	0.74	−0.049 ± 0.035	0.25
3,4-diCl	H	*l*	5	1.03	0.76	0.71	−0.163 ± 0.037	<0.02
3,4-diCl	CH_3	*dl*	6	0.88	0.83	0.68	−0.098 ± 0.024	0 01
3,4-diCl	CH_3	*d*	6	0.75	1.05	0.63	−0.155 ± 0.077	0.10
3,4-diCl	CH_3	*l*	7	0.86	0.68	0.56	−0.151 ± 0.048	<0.05

here are the long pauses that occasionally followed a reinforcement and protracted periods of time when no response occurred following return to the experimental cage.

With the chloro-substituted compounds, the rat's response rate appeared irregularly depressed. Pauses between and after reinforcement became more frequent and were shorter than those with the unsubstituted amphetamines. These phenomena present an overall "scalloped" effect on the cumulative records. The depression of rate appeared more pronounced with the larger doses. A detailed comparison of segments of the records showing the differences between normal running rates, rates with *dl*-amphetamine, and rates with *dl*-1-(2,4-dichlorophenyl)-2-aminopropane is presented in Fig. 3.

The effects of the compounds on the response-rate ratios are shown in Table II. The average response-rate ratios of individual rats were computed for each dose of each drug. The slope values are relative, based on three equally spaced doses and do not represent numerically the dose-response slopes (*cf.*, Table I for actual doses used). They serve the purpose of indicating whether or not there was a change in the rate ratio and whether or not the change was significant. With *dl*- and *d*-amphetamine and methamphetamine, the rats produced lower than normal rates but showed little change from dose to dose. The chloro-substituted compounds, with the exception of *d*-1-(3,4-dichlorophenyl)-2-aminopropane, caused the rats to work at rates that decreased significantly as the doses were increased.

As shown in Table III, the length of time to complete the 40-reinforcement sessions with all of the drugs studied was longer than normal. The means and standard errors of the time ratios are presented for each dose of each drug. In general, the mean time to complete a session increased as the dose was increased. The increases appeared greatest with *dl*- and *d*-amphetamine, methamphetamine, the 4-chlorophenyl, and the 2,4-dichlorophenyl compounds. The variability among animals was high as evidenced by the rather large standard error terms.

DISCUSSION

In animals trained on FR procedures for food reinforcement the response rate normally will remain unchanged once it is established. The development of post-reinforcement pauses, however, is dependent upon several controllable variables (7, 8). If the size of the FR is increased, *e.g.*, from 30 to 100 responses, post-reinforcement pauses become pronounced and in some instances the rat stops responding entirely. If the size of the reinforcement is decreased, the post-reinforcement pauses become more frequent and of greater duration. The state of

TABLE III.—MEAN TOTAL TIME RATIOS

Compound			Low Dose	Middle Dose	High Dose
Phenyl Substituent	Amino Substituent	Isomer	Mean Ratio, ± S.E.	Mean Ratio, ± S.E.	Mean Ratio, ± S.E.
H	H	*dl*	1.74 ± 0.33	3.56 ± 0.80	6.83 ± 2.03
H	H	*d*	2.27 ± 0.37	10.32 ± 2.56	20.05 ± 4.64
H	CH_3	*d*	4.46 ± 1.82	9.33 ± 2.89	16.54 ± 4.76
4-Cl	H	*dl*	1.64 ± 0.26	8.72 ± 3.07	10.28 ± 3.34
4-Cl	H	*d*	1.20 ± 0.16	6.04 ± 2.35	11.30 ± 6.04
4-Cl	H	*l*	1.24 ± 0.13	2.17 ± 0.16	8.40 ± 2.66
2,4-diCl	H	*dl*	1.77 ± 0.10	2.79 ± 0.10	17.76 ± 4.10
2,4-diCl	H	*d*	4.09 ± 1.99	6.59 ± 2.30	12.24 ± 5.31
2,4-diCl	H	*l*	1.27 ± 0.18	11.59 ± 4.39	13.14 ± 4.20
3,4-diCl	H	*dl*	1.39 ± 0.22	2.28 ± 0.71	3.65 ± 1.30
3,4-diCl	H	*d*	1.81 ± 0.75	3.76 ± 1.99	2.25 ± 0.90
3,4-diCl	H	*l*	1.03 ± 0.88	3.07 ± 1.63	3.63 ± 2.19
3,4-diCl	CH_3	*dl*	1.42 ± 0.30	1.52 ± 0.38	2.74 ± 1.00
3,4-diCl	CH_3	*d*	1.68 ± 0.45	1.04 ± 0.12	1.77 ± 0.21
3,4-diCl	CH_3	*l*	1.35 ± 0.21	1.94 ± 0.43	3.89 ± 1.20

deprivation and/or satiation of the animal influences the character of the post-reinforcement pause, *i.e.*, the more satiated the animal the longer the pauses. Generally, the response rates remain relatively unchanged despite the length of the post-reinforcement pause. The phenomenon of pausing has been termed "fixed-ratio strain" (4). Rats with stable FR behavior showing little or no ratio-strain responded with frequent and sometimes very long pauses when under the influence of amphetamine compounds. The overall effect of the amphetamines in this situation was considered to be a lowering of the threshold for fixed-ratio straining that resembled, in many respects, a premature onset of satiation.

The chloro-substituted compounds used in this study depressed the rats' FR behavior by causing an irregular decline in response rate that appeared different from the depression seen with the unsubstituted amphetamines as shown in Fig. 3. The cumulative record data indicate numerous relatively short pauses occurred with the slowed response rates. Even segments of the cumulative records that appear as long pauses, on closer inspection, reveal the presence of occasional short bursts of 2–3 or more lever presses. This random, intermittent responding is illustrated in part *D* of Fig. 3. At the doses used, the 4-chlorophenyl and 2,4-dichlorophenyl compounds produced the most marked depression of the FR behavior in regard to response rate and prolongation of total time to complete sessions.

Except for the 1-(4-chlorophenyl)-2-aminopropane compounds, the *l* isomers of the chloro compounds appeared more potent than the *dl* and *d* members. This observation suggests that the presence of dichloro substitutions on the phenyl ring reverses the relative potency of the isomers seen with *d*- and *l*-amphetamine as reported in a previous study (4). Monochloro substitution on the 4 position seemingly failed to cause this reversal since the *d*-1-(4-chlorophenyl) compound appeared more potent than either of the *dl* or *l* compounds.

ADDENDUM

None of the new compounds tested could be considered a clinically useful drug.[2] In one double blind study with 16 moderately obese subjects, 20 and 30 mg. of *l*-1-(2,4-dichlorophenyl)-2-aminopropane was compared with placebo and a marketed anorectic drug. When the patients received the chlorinated compound they reported poor appetite control. Several patients mentioned a sense of agitation associated with inability to work effectively; others complained of nausea, nightmares, irritability, and lack of tolerance for the usual difficulties at work and at home. In general, both the patient and his family disapproved of the experimental compound. The placebo and known drug were well tolerated and the active known compound could be shown to cause significant weight loss in a 1-week period. No patient preferred the chlorinated compound but 15 preferred the active drug to placebo. It would appear that the pseudo-satiation-type of suppression of the FR preformance seen after the rats were given amphetamine is consistent with suppression of appetite clinically. The decrease of rate with the scalloped appearance resulting from frequent short pauses seems to indicate a different, and probably undesirable, type of clinical activity.

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(8) Sidman, M., and Stebbins, W. C., *J. Comp. Physiol. Psychol.*, **7**, 114(1954).

[2] A preliminary trial of some of these appetite suppressants was conducted by Dr. S. M. Chernish of the Department of Clinical Research, Eli Lilly and Co.

Psychopharmacological Studies of Some 1-(Chlorophenyl)-2-aminopropanes II

Effects on Avoidance and Discrimination Behavior

By JOHN E. OWEN, JR.

A series of 1-(chlorophenyl)-2-aminopropanes previously shown to suppress appetitive-controlled behavior were compared with *d*-amphetamine and methamphetamine on an avoidance procedure. Rats trained to press a lever to avoid intermittent electroshocks were used. In alternate 10-minute periods, a rat received a time-out (TO) when no responding on the lever was necessary. The discrimination between the two periods was cued by appropriate visual stimuli. The two lowest doses that suppressed the appetitive-controlled behavior were studied. With the unsubstituted amphetamines, the rats produced high response rates during both the avoidance and TO periods with a reduction in the number of shocks received. On the whole, the chloro-substituted compounds produced relatively small increases in the rats' response rates with little or no change in the number of shocks received. These results indicate that, at the doses used, the chloro-substituted compounds produced less CNS stimulation than did *d*-amphetamine or methamphetamine.

AN OPERANT-BEHAVIOR avoidance procedure originally described by Sidman (1) has been employed to study the effects of drugs of the amphetamine class on conditioned avoidance responding (2–5). Rats, trained to press a lever (respond) to avoid or postpone brief, intermittent electroshocks, produced higher than normal response rates with the drugs in this study. Verhave (4) introduced a time-out contingency (TO) to the Sidman procedure. Rats were trained to avoid electroshock in the presence of a 433-c.p.s. tone stimulus. They were then taught to discriminate between tone-on avoidance periods and tone-off TO periods when no electroshocks would occur and responding was not necessary. As the discrimination developed the rats did not respond in the absence of the tone stimulus. With the amphetamines the animals had a tendency to continue responding during the TO. This effect with the drugs was considered as a breakdown of the tone stimulus control that maintained the acquired discrimination. In a previous study Verhave (3) had shown that, in the absence of established avoidance behavior, methamphetamine did not cause rats to produce high response rates with or without shock.

Rats given drugs of the amphetamine type show stimulated avoidance behavior only in a limited range of doses (3, 6). Doses above certain optimal levels (the level depending on individual variation from rat to rat) cause the animals to produce erratically depressed response rates. In some cases responding ceases entirely and the animal dies at much less than normally toxic doses of the drugs with normally nonlethal electroshock. Weiss, *et al.* (7), in a study of this phenomenon reported that rats and mice exposed to aversive electroshock died at much lower doses of amphetamine than animals that were not shocked. Also, the mode of death appeared different from that seen with lethal doses of the drug.

The investigation reported here utilized a Sidman-type avoidance TO procedure to study the influence of some 1-chlorophenyl-2-aminopropanes and their optical isomers on avoidance and discrimination behavior. These compounds were reported (8) to suppress appetitive-controlled fixed-ratio behavior. This study was undertaken as an extension of Rathbun's observations (9) of these compounds in mouse activity studies. He found that, although the 1-(4-chlorophenyl)-2-aminopropane compounds were more active central nervous system stimulants than the dichloro compounds, all caused little or no increased activity in the mice at dose ranges that induced appetite suppression in rats on food-consumption, weight-loss studies.

EXPERIMENTAL

Materials.—The compounds and doses used are listed in Table I. The compounds were dissolved in distilled water and administered subcutaneously.

Methods.—The subjects were six male rats of the Long-Evans strain, weighing 300–450 Gm. By means of a modification of the procedure described by Sidman (1) they were trained to press a lever to avoid brief intermittent electroshocks.

Two experimental cages were used in this investigation. Each measured 10 × 5 × 9 in. and was designed with four electrically isolated metal walls and a grid floor made of parallel $^3/_{16}$-in. diameter stainless steel rods. A response lever, a

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TABLE I.—DRUGS AND DOSES USED IN AVOIDANCE TESTING

Drug	Doses, mg./Kg. Low	High
d-Amphetamine sulfate	1.0	2.0
d-Methamphetamine HCl	1.0	2.0
dl-1-(4-Chlorophenyl)-2-aminopropane HCl	2.0	3.2
d-1-(4-Chlorophenyl)-2-aminopropane HCl	1.0	2.0
l-1-(4-Chlorophenyl)-2-aminopropane HCl	2.0	3.2
dl-1-(3,4-Dichlorophenyl)-2-aminopropane HCl	2.0	5.0
d-1-(3,4-Dichlorophenyl)-2-aminopropane HCl	3.2	5.0
l-1-(3,4-Dichlorophenyl)-2-aminopropane HCl	2.0	3.2
dl-1-(2,4-Dichlorophenyl)-2-aminopropane HCl	3.2	5.0
d-1-(2,4-Dichlorophenyl)-2-aminopropane HCl	3.2	5.0
l-1-(2,4-Dichlorophenyl)-2-aminopropane HCl	2.0	3.2
dl-1-(3,4-Dichlorophenyl)-2-methylaminopropane HCl	3.2	5.0
d-1-(3,4-Dichlorophenyl)-2-methylaminopropane HCl	3.2	5.0
l-1-(3,4-Dichlorophenyl)-2-methylaminopropane HCl	2.0	3.2

modified telephone-type switch (10), was mounted on one end wall of each cage. A small 24-volt pilot light was mounted in the wall above the lever and another below the level of the grid floor. The cages were contained in light-proof, sound-resistant, ventilated boxes isolated from the control equipment.

Brief electroshocks of 0.2-second duration were given to the rat through the grid floor and walls of the cage at 2-second intervals (SS). A response on the lever postponed the next shock for 20 seconds (RS). Responses within this 20-second interval produced another 20 seconds without shock. Thus, with a steady rate of responding the rat avoided being shocked. The shock was provided by a constant-current generator passing half-wave 60-cycle d.c. at 1 milliamp. The shock was delivered to the cage through a "grid scrambler" that, in a random fashion, rapidly changed the current polarity on the individual grid rods and walls. The rat was thus unable to avoid shock by standing on rods or leaning against a wall of similar polarity.

Appropriate electrical relay circuits and timers were used to control the procedure and record the data on impulse counters and cumulative recorders.

After several training sessions, when the rats began developing stable avoidance behavior, the TO contingency was introduced. During alternate 10-minute periods the light over the lever went out and the light below the cage floor came on, signifying the TO when no shocks would occur. The rats learned to discriminate between the avoidance

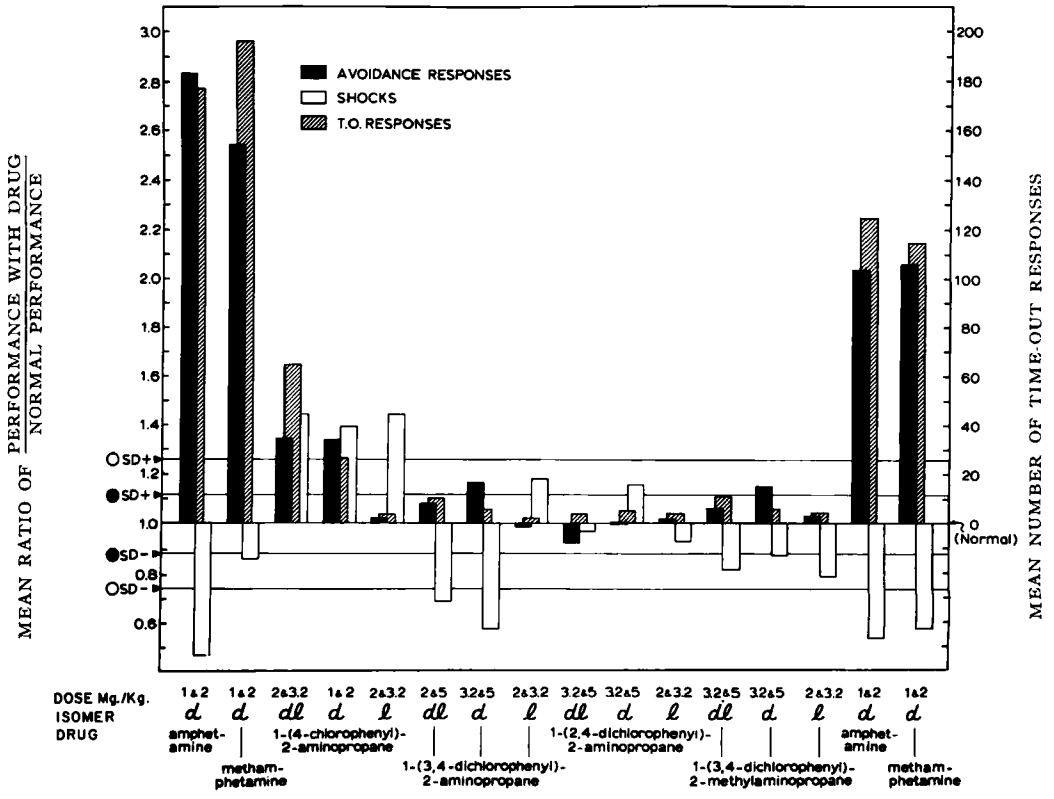

Fig. 1.—Summary of combined dose drug effects of compounds used in this study on avoidance discrimination behavior.

periods and TO by the change in the source of cage illumination. As the rats developed discrimination they no longer responded during the TO. Each avoidance-TO session lasted for 3 hours. When drugs were tested, each rat was given a 1-hour "warm-up" before drug administration. The animal then was returned to the experimental cage for a 3-hour session.

Only the two lowest doses of each compound that suppressed appetitive-controlled fixed-ratio behavior were used (8). *d*-Amphetamine and methamphetamine were tested at the beginning and again at the end of the investigation. The chloro-substituted compounds were run only once at each dose in a random order. Non-drug sessions run at 24 and 48 hours after a drug session failed to show residual drug activities on either avoidance or TO behavior. Thus, a time lapse of at least 48 hours was allowed between sessions for the individual animals.

Data.—The number of avoidance responses, the number of responses during TO, and the number of shocks received were recorded for each hour of each session. The means and standard deviation of avoidance responses and shocks received for the second hour of 11 non-drug sessions that were run during the study were calculated for each rat. Only non-drug sessions were used when at least 48 hours had elapsed since a previous drug session. The avoidance response and shock data from the second hour after drug administration of each drug session were used to calculate ratios of performance with drug to normal performance. Since TO responding normally was nonexistent in non-drug runs, no ratios were determined for this contingency of the procedure. Only the actual number of responses was counted.

Cumulative records of responses were made of each session to provide graphic data of the character and distribution of the responses and shocks.

RESULTS

The results of this investigation have been summarized by combining the data from both doses of each compound—presented in Fig. 1 as means of the ratios of avoidance responding and shocks received and the means of the TO responses. The data were combined because relatively little difference was seen between dose levels of each compound. This was especially true of the chloro-substituted drugs. The normal baseline (ratio of 1.0) and the means of normal standard deviations of the avoidance responses and shocks received have been included in Fig. 1.

At the beginning and at the end of the study with *d*-amphetamine and methamphetamine, the rats showed increased avoidance and TO responding with a decrease in the number of shocks received. With the *dl* and *d*-1-(4-chlorophenyl)-2-aminopropane

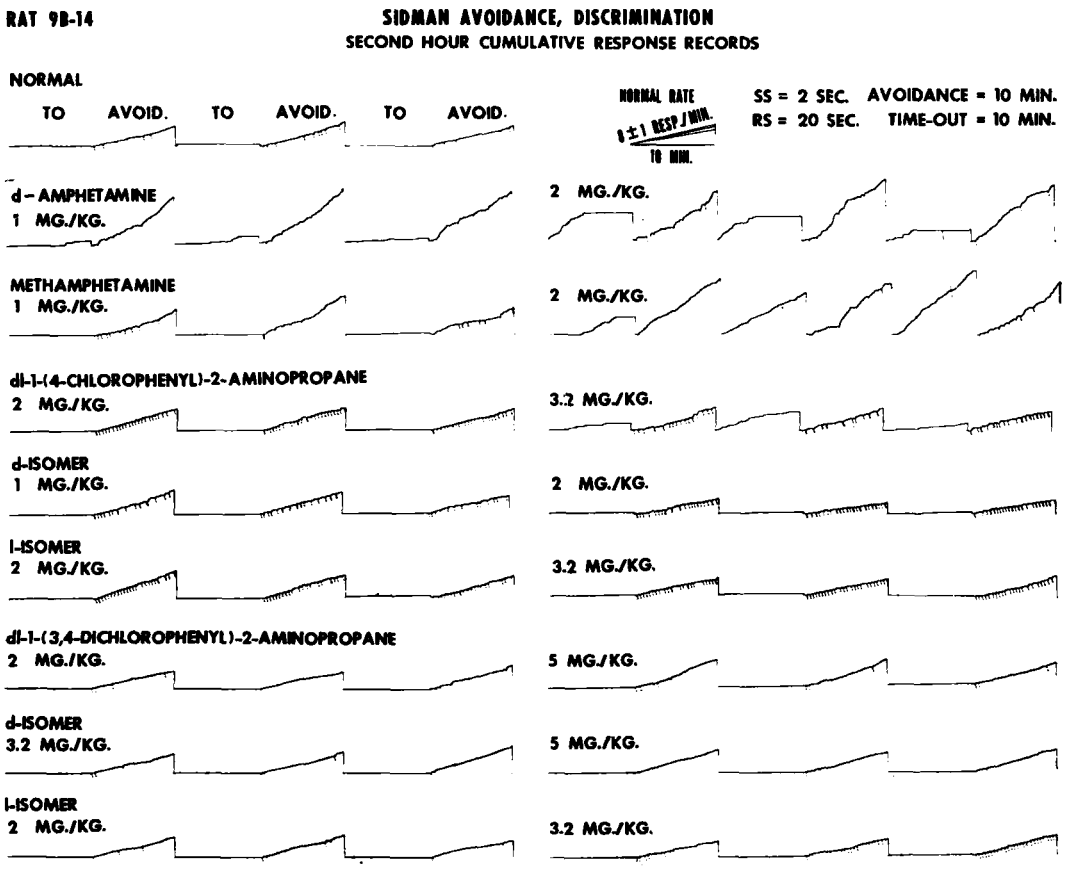

Fig. 2.—Cumulative response records of a rat comparing effects of drugs on avoidance discrimination behavior to normal behavior.

rats showed increased responding during the avoidance and TO periods with an increase in the number of shocks received. The magnitude of the response increases appeared to be one-third to one-half that of the des-chloro-compounds. The *l* isomer showed a marked increase only in the ratios of shocks received. Except for the *dl* and *d*-1-(3,4-dichlorophenyl)-2-aminopropanes, the remainder of the compounds produced ratios that fell well within the limits of normal standard deviation of avoidance responses and shocks, and near the normal of TO responding. The shock ratios of the *dl* and *d*-1-(3,4-dichloro)-2-aminopropane compounds appeared below the lower standard deviation limit.

Figures 2 and 3 show the cumulative response records of one typical rat. Included are examples of records of the second hour of non-drug sessions and records of the second hour after drug administration of *d*-amphetamine and methamphetamine and the 12 chloro-substituted compounds. This rat had a normal avoidance response rate of 8 ± 1 responses per minute, which has been drawn in the figures as the normal rate. The short vertical pen marks on the avoidance segments indicate points when the animal was shocked. Significant TO responding in these records appeared only after the administration of *d*-amphetamine, methamphetamine, and the higher doses of *dl* and *d*-1-(4-chlorophenyl)-2-aminopropane. The increased avoidance responding was apparent with the administration of the amphetamine compounds. The stimulated avoidance responding can be described as erratic and is illustrated with the *d*-amphetamine and methamphetamine and the 3.2 mg./Kg. dose of the *dl*-1-(4-chlorophenyl)-2-aminopropane. The animal received several successive shocks with only one or two responses between shocks and then responded in very rapid bursts of lever pressing. The overall effect produced very irregular appearing cumulative response records.

DISCUSSION

Data from this study have confirmed Verhave's observations (4) that the administration of amphetamine compounds to rats that have well developed avoidance and discrimination behavior causes the animals to show a stimulation of avoidance responding with a loss of ability to discriminate between the two contingencies of the procedure. The *dl* and *d*-1-(4-chlorophenyl)-2-aminopropane, unlike the dichloro-substituted compounds, more nearly resembled *d*-amphetamine and methamphetamine with regard to the rats' avoidance and TO responding. The magnitude of effect was somewhat less. However, qualitatively the *dl* and *d*-4-chloro compounds differed from all of the others since the animals received more shocks in spite of the increased avoidance responding. The *l* isomer of the 4-chloro compounds also showed high shock ratios

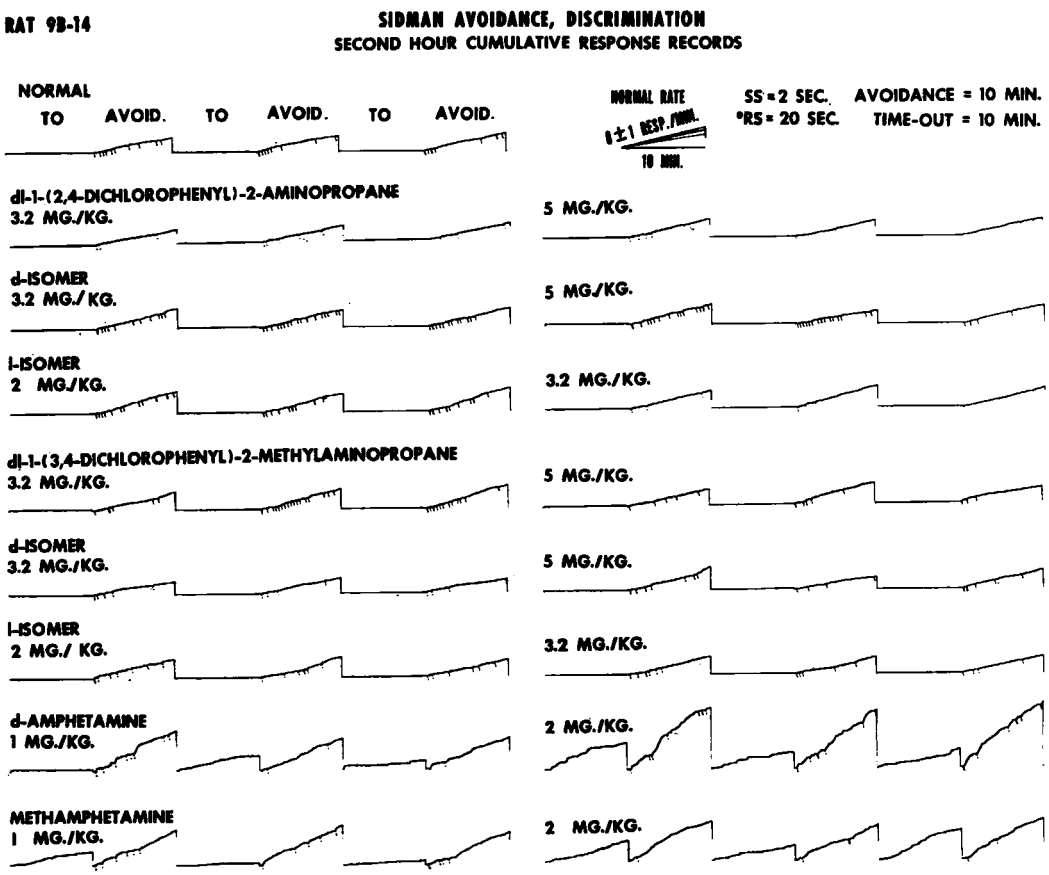

Fig. 3.—Cumulative response records of a rat comparing effects of drugs on avoidance discrimination behavior to normal behavior.

in the rats. Dichloro substitutions on the 2,4 and 3,4 positions of the phenyl ring of 1-phenyl-2-aminopropane structures, at the doses used, inhibited the behavioral stimulation effects seen with the parent structure. The general lack of responding during the TO contingency after administration of the dichloro-substituted compounds showed that the stimulus control of the changing light sources was maintained. The lowered shock ratios seen in the rats with the *dl* and *d*-1-(3,4-dichlorophenyl)-2-aminopropanes could be termed as an increase in efficiency since the avoidance ratios were only slightly raised, *i.e.*, more protection for the same work output.

Although the *d*-amphetamine and methamphetamine response rates were approximately 30% less at the end than at the start of the study, the mean avoidance response-rate ratios and TO responses with the chloro-substituted compounds were well below those of the amphetamine compounds. For the most part, the chloro compound data fell close to or within the normal standard deviation limits. The difference in the amphetamine data could be due to a development of tolerance to the 1-phenyl-2-aminopropane structure or more probably to a gradual strengthening of the behavior over the period of time covered by the investigation to provide a resistance to drug action.

SUMMARY

Some 1 chlorophenyl-2-aminopropanes have been compared with *d*-amphetamine and methamphetamine for effects on avoidance and discrimination behavior in rats. The doses used were the two lowest that caused suppression of an appetitive-controlled behavior.

After *d*-amphetamine, methamphetamine, and *dl* and *d*-1-(4-chlorophenyl)-2-aminopropane, rats showed increased avoidance response rates as well as responding during time-out periods when it was not necessary. This latter effect was considered as a loss of ability to discriminate. The rats when given dichlorophenyl-2-aminopropanes showed little or no change from normal in avoidance or discrimination behavior.

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Alkaloids of *Vinca rosea* Linn. (*Catharanthus roseus* G. Don) XV

Analysis of Vinca Alkaloids by Thin-Layer Chromatography

By NANCY J. CONE, RUTHANNE MILLER, and NORBERT NEUSS

Thin-layer chromatography of various Vinca alkaloids is described. Adsorbents, solvent systems, and their influence on the R_f values are discussed. R_f values for 26 Vinca alkaloids are given.

THE CLINICAL use of vinblastine[1] (2), a member of a new class of oncolytic alkaloids (3) from the ornamental shrub *Vinca rosea* Linn. (*Catharanthus roseus* G. Don), prompts us to publish the results of the use of thin-layer chromatography (TLC) in the identification of this and other alkaloids obtained from this plant in our laboratories (4). The laborious fractionations and repeated chromatographies required in the preparation of several of these alkaloids were greatly facilitated by continuously monitoring fractions with TLC. The technique was also found to be extremely useful in the course of chemical work leading to the structure elucidation of catharanthine (5) and vindoline (6) as well as the dimeric alkaloids vinblastine (VLB) and leurocristine[2] (LCR) (7). Finally, the behavior of compounds on TLC was often found to be a most efficient criterion of purity. This was especially true in the case of several dimeric alkaloids (8).

The advantages of TLC over paper chromatography, however, should not prevent us from mentioning some of the rules of paper chromatographic technique which also apply here. One cannot, for example, use indiscriminantly the results obtained on pure compounds to identify these components in a crude mixture of alkaloids or complex reaction mixtures without using

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[2] The generic name of leurocristine is vincristine.

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TABLE I.—SOLVENT SYSTEMS

System No.	Components
1	Alumina Chloroform-Ethyl Acetate (1:1)
2	Silica Ethyl Acetate-Absolute Ethanol (3:1)
3	Alumina Ethyl Acetate-Absolute Ethanol (3:1)
4	Alumina Benzene (100%)
5	Alumina Chloroform (100%)
6	Silica Chloroform (100%)
7	Alumina Benzene-Chloroform (3:1)
8	Silica Ethyl Acetate-Absolute Ethanol (1:1)
9	Silica 100% Ethyl Acetate
10	Alumina 100% Ethyl Acetate
11	Silica, prepared using 0.5 N KOH instead of H_2O Ethyl Acetate-Absolute Ethanol (1:1)

several solvent systems, different adsorbents, and appropriate developing reagents. Rules similar to those in paper chromatography apply also to the determination of R_f values, preparation of chambers, etc. Finally, additional criteria of identification by various physical methods should always be supplemental whenever this is practical.

Since a bibliography on TLC, which also includes several excellent reviews (9), deals with practically all the phases of this technique and its applications, we shall limit our discussion to a description of the experimental details necessary for the reproducibility of our results.

Preparation of Plates

Alumina.—Our studies were conducted on plates prepared with Fluka[3] special alumina for thin-layer chromatography. Thirty grams of this adsorbent were shaken with 75 ml. of distilled water for 30 seconds, poured into the Desaga[4] applicator, and spread on plates. They were then allowed to dry in the air for about 1 hour and activated in an oven at 110–120° for 15 minutes. Immediately afterward, the plates were allowed to cool in a desiccator and stored for varying lengths of time in a wooden cabinet over drying reagents (Drierite and calcium chloride).

Silica.—The plates were prepared using Merck[4] silica gel in the following manner: Twenty-five grams of the adsorbent was shaken with 50 ml. of distilled water for 30 seconds and applied using the applicator. They were used after having been allowed to air dry for at least 2 hours. These plates are also stored in a wooden cabinet over drying reagents.

Application of Alkaloids

Most of our studies, except where noted, were done using a concentration of 50 mcg. of alkaloid per spot (5 μl. of a 10 mg./ml. solution) in a suitable solvent (in most cases CH_2Cl_2 or $CHCl_3$).

When mixtures of alkaloids were investigated, quantities of 100–200 mcg. were used. Spots were marked 1.5 cm. from the lower edge of the plates. No more than nine samples were applied per plate (10 × 10 cm.).[4]

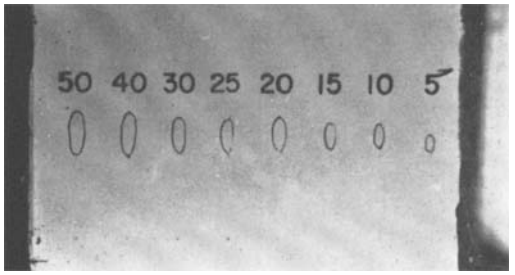
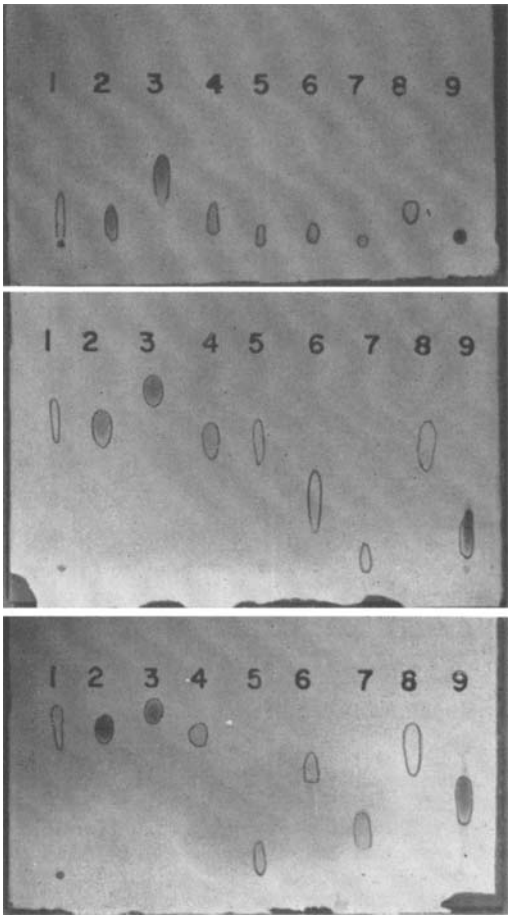


Plate 1—Different concentrations of catharanthine (5–50 mcg): adsorbent silica; solvent system, ethyl acetate.



Plates 2, 3, 4—Dimeric alkaloids on silica adsorbent. Key: carosidine *1*, catharicine *2*, carosine *3*, catharine *4*, pleurosine *5*, neoleurocristine *6*, neoleurosidine *7*, vincarodine *8*, vindolidine *9*. Solvent systems used were ethyl acetate for plate 2 (top); ethyl acetate–ethanol (3:1) for plate 3 (middle); and ethyl acetate–ethanol (1:1) for plate 4 (bottom).

[3] Available from Gallard-Schlesinger Chemical Manufacturing Corp.
[4] Available from Brinkmann Instruments.

TABLE II.—R_f VALUES OF VINCA ALKALOIDS IN DIFFERENT SOLVENT SYSTEMS

	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9	Color: Ceric Ammonium Sulfate
Ajmalicine	0.57	0.68	0.72	0.03	0.51	0.02	0.09	0.68	0.54	Yellow
Carosidine	...	0.58	...	...	...	...	...	0.59	0.10	Yellow
Carosine	...	0.71	...	...	...	...	...	0.65	0.24	Purple-gray
Catharanthine	0.77	0.59	0.74	0.12	0.77	0.03	0.37	0.58	0.38	Green (fades quickly)
Catharine	0.18	0.58	0.76	...	...	...	...	0.56	0.10	Yellow
Catharosine	...	0.56	...	...	...	...	...	0.58	0.08	Purple
Isoleurosine	0.35	0.22	...	0.00	0.23	0.00	0.00	...	...	Gray
Leurosine	0.27	0.35	...	0.00	0.20	...	...	...	...	Gray
Lochnericine	0.15	0.25	0.77	...	...	...	...	0.46	0.03	Blue
Lochneridine	0.00	0.00	0.21	...	...	...	...	0.04	0.00	Blue-green
Lochnerine	0.04	0.35	0.70	...	...	...	...	0.42	0.06	Pale gray
Neoleurocristine	...	0.27	...	...	...	...	...	0.43	0.03	Blue
Neoleurosidine	...	0.06	...	...	...	...	...	0.17	0.00	Yellow-brown
Perivine	0.05	0.30	0.48	...	...	...	...	0.39	0.11	Lt. brown
Pleurosine	...	0.51	0.42	...	...	...	...	0.07	0.03	Yellow
Serpentine	0.03	0.00	0.11	...	...	...	...	0.00	0.00	
Tetrahydroalstonine	0.76	0.60	0.73	0.05	0.66	0.04	0.29	0.76	0.69	Yellow-green
Vinblastine	0.25	0.24	0.66	0.00	0.17	0.00	0.00	0.33	0.04	Purple
Vincamicine	0.03	0.09	0.42	...	...	...	...	0.20	0.00	Bluish orange
Vincarodine	...	0.50	...	...	...	...	...	0.50	0.10	Blue (fades quickly)
Vindolicine	0.24	0.46	0.73	...	...	...	...	...	...	Blue
Vindolidine	...	0.15	...	...	...	...	...	0.29	0.00	Blue
Vindoline	0.44	...	0.68	0.00	0.53	0.03	0.06	0.57	0.20	Crimson
Vindolinine	0.55	0.37	0.70	0.00	0.51	0.00	0.09	0.44	0.13	Orange
Virosine	0.09	0.54	0.63	...	...	...	...	0.48	0.09	Colorless
Sitzirikine	...	0.31	...	...	...	...	...	0.45	0.09	Yellow-green

TABLE III.[a]—SEPARATION OF VARIOUS VINCA ALKALOIDS

	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)	(I)	(J)	(K)	(L)	(M)	(N)	(O)	(P)	(Q)
Ajmalicine (A)	...	1	1	1	1	1	1	1	1	1	1	1,3	1	1	7	7	1
Catharanthine (B)	1	...	1	1	1	1	1	1	1	1	2	1,3	1	1	1	1	1
Catharine (C)	1	1	...	1	...	3	1	1	1	1	1	4	1	3	1	1	6
Isoleurosine (D)	1	1	1	...	5	...	1	...	1	1	1	3	...	...	7	7	3
Leurosine (E)	1	1	...	5	...	...	1	...	1	1	1	3	...	...	7	7	3
Lochnericine (F)	1	1	3	...	...	...	1	3	6	1	1	3	1	3	1	1	3
Lochneridine (G)	1	1	1,3	1	1	1	...	3	3	6	1	1,3	3	1	1	1	3
Lochnerine (H)	1	1	1,3	...	...	3	3	...	6	3	1	...	3	...	1	1	3
Perivine (I)	1	1	1	1	1	6	6	6	...	3	1	1	3	6	1	1	6
Serpentine (J)	1	1	1	1	1	1	1,6	3	3	...	1	1	6	1	1	1	6
Tetrahydroalstonine (K)	1	2	1	1	1	1	1	1	1	1	...	1	1	1	1	1	1
VLB (L)	1,3	1,3	4	3	3	3	3	...	1	1	1	...	...	4	...	...	3
Vincamicine (M)	1	1	1	...	...	1	3	3	3	6	1	...	...	3	1	1	3
Vindolicine (N)	1	1	3	...	...	3	1	...	6	1	1	4	3	...	1	1	...
Vindoline (O)	7	1	1	7	7	1	1	1	1	1	1	...	1	1	...	3	1
Vindolinine (P)	7	1	1	7	7	1	1	1	1	1	1	...	1	1	3	...	1
Virosine (Q)	1	1	6	3	3	3	3	3	6	6	1	3	3	...	1	1	...

[a] Numbers refer to solvent systems listed in Table I.

Development of Plates

One-hundred milliliters of the developing solvent system were placed in the bottom of the chamber to insure a 5–7 mm. layer of the solvent. Two 15 × 25-cm. rectangular pieces of Whatman No. 1 filter paper were immersed in the solvent and placed on the two long sides of the chamber. Proper saturation of the system was assured by waiting 30 minutes before using the chamber. After this time the plates were placed in the chamber and rested on a 50-ml. graduate cylinder as a support. Time of development (when the solvent front reached a line marked 10 cm. from the point of application) was about 30 minutes for silica plates and 45 minutes to 1 hour for alumina plates.

TABLE IV.—SEPARATION OF CLOSELY RELATED DIMERIC ALKALOIDS

Compounds	Suitable Systems for Separating
Leurosine VLB Isoleurosine	No. 2 or No. 8
Leurosidine Leurocristine VLB or Leurosine	Develop first in No. 10; air dry 10 min.; then develop in No. 3
Leurosidine Sulfate Leurocristine Sulfate VLB Sulfate or Leurosine Sulfate	No. 11

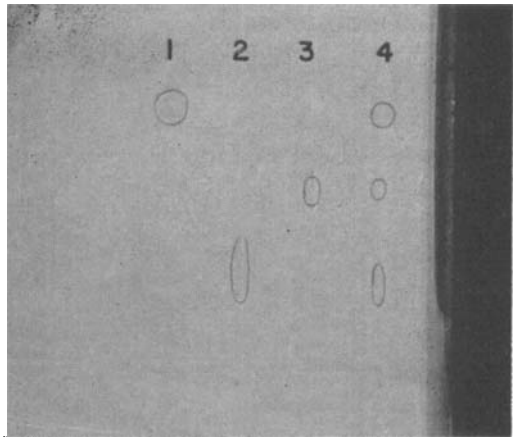


Plate 5.—Oncolytic alkaloids on alumina adsorbent (See Table IV for solvent system.) Key: VLB *1*, leurosine *2*, leurocristine *3*, and mixture *4* of 25 mcg. each of *1*, *2*, and *3*.

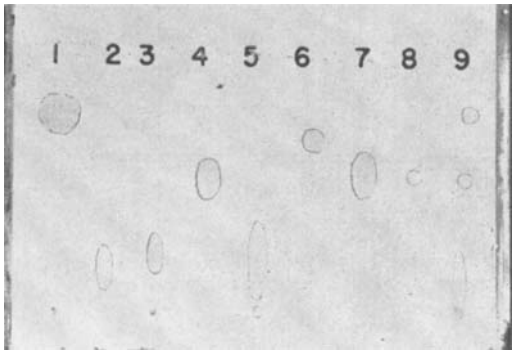


Plate 6.—Monitoring of elution chromatography on alumina. Comparison of an early fraction with several alkaloids known to occur in alkaloidal extract (Fraction A) (4). Key: catharanthine *1*, VLB *2*, isoleurosine *3*, ajmalicine *4*, leurosine *5*, tetrahydroalstonine *6*, vindoline *7*, vindolinine *8*, fraction No. 2 from fraction A *9* (100 mcg.).

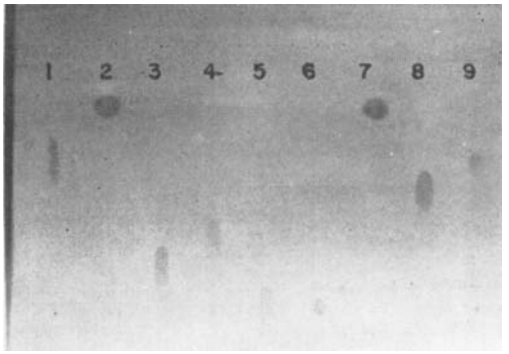


Plate 7.—Examples of results from Table IV on alumina with a chloroform-ethyl acetate (1:1) solvent system. Key: ajmalicine *1*, catharanthine *2*, catharine *3*, leurosine *4*, perivine *5*, serpentine *6*, tetrahydroalstonine *7*, vindoline *8*, vindolinine *9*.

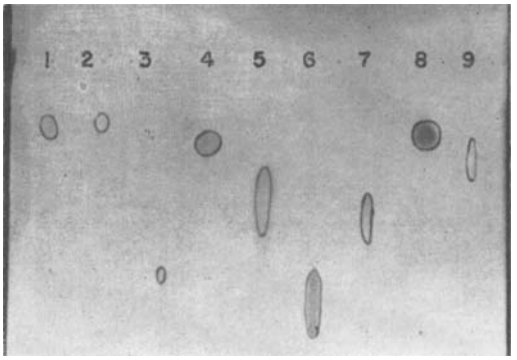


Plate 8.—Examples of results from Table IV on silica with a ethyl acetate-absolute ethanol (3:1) solvent system. Key: catharine *1*, lochnericine *2*, lochneridine *3*, lochnerine *4*, perivine *5*, serpentine *6*, vincamicine *7*, vindolicine *8*, virosine *9*.

Solvent Systems

The solvent systems used in this study are listed in Table I.

Alkaloidal Standards

The alkaloids[5] used in our study had physical characteristics in accordance with those described in the literature (4) and the "Lilly Collection of Physical Data of Indole and Dihydroindole Alkaloids" (10).

Spraying Reagents

Dragendorff's Reagent.—A stock solution is prepared using 2.6 Gm. of bismuth subcarbonate, 7.0 Gm. of dry sodium iodide, and 25 ml. glacial acetic acid. This solution is boiled about 4 minutes and allowed to cool overnight. It is then decanted or filtered from any insoluble material. For every 25-ml. solution obtained, 100 ml. ethyl acetate is added to make the stock solution. The reagent is made fresh daily with 24 ml. ethyl acetate, 10 ml. glacial acetic acid, 4 ml. stock solution, and 2 ml. distilled water, added dropwise.

Ceric Ammonium Sulfate Reagent.[6]—A 1% solution is prepared by dissolving 1 Gm. of ceric ammonium sulfate in 99 Gm. of syrupy phosphoric acid. It is necessary to heat the mixture on a hot plate 5–10 minutes before solution is complete.

DISCUSSION OF RESULTS

The results are tabulated in Tables II, III, and IV. Table II is self-explanatory. From Tables III and IV one can find a system and an adsorbent suitable for the separation of given alkaloids. As far as the reproducibility of R_f values is concerned, we noted that it varies with differences in concentration (Plate 1), the nature of the mixture, the quality of plates used, temperature, and related factors. This has been observed before by Stahl and others (9).

A few examples were chosen which indicate an efficient separation of certain dimeric alkaloids (see Plates 2–5). Plate 6 illustrates the use of thin-layer chromatography to monitor chromatographic fractions. Plates 7 and 8 are representative of the chromatograms which were used to compute the R_f values found in Tables II and III.

[5] We thank Drs. G. H. Svoboda and M. Gorman for authentic samples of several of the alkaloids used in this study and Mr. W. E. Kruse for art and photographic work.

[6] This reagent was introduced for the analysis of Vinca alkaloids by I. Jakovljevic, from the Lilly Control Laboratories, whom we thank for permission to use it in our studies.

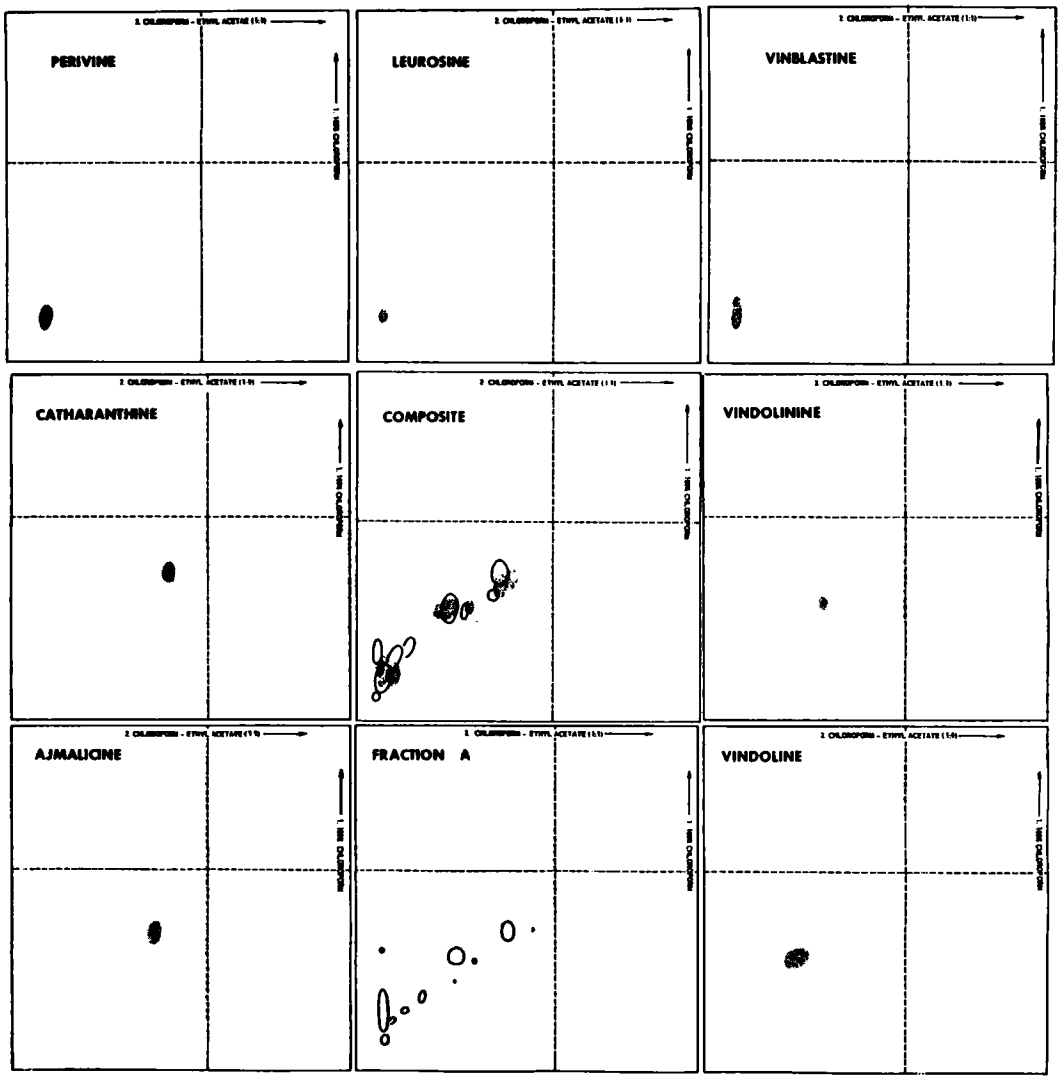


Fig. 1.—Didimensional chromatography on alumina using 1, chloroform (100%) and 2, chloroform-ethyl acetate (1:1) solvent systems. The concentration of Fraction A was 100 mcg. and for the composite 50 mcg. of the following alkaloids applied: ajmalicine, catharanthine, leurosine, perivine, VLB, vindoline, and vindolinine.

When complex mixtures (*e.g.*, Fraction A) (4) were subjected to solvent systems Nos. 1–6 in one dimension, separation was unsatisfactory. A better resolution was obtained by the use of didimensional chromatography on either alumina or silica.

Comparison of the position of spots from individual alkaloids in didimensional chromatograms with the position of spots in the mixture, run under identical conditions, gives a good indication of the nature of the mixture's different components. This procedure is illustrated in Fig. 1. The use of the ceric ammonium sulfate reagent, which gives specific color reactions, was also found to be very useful in this particular technique.

The figure, however, also illustrates that it is not possible to identify spots in the composite unequivocally. Even under similar conditions the location of spots is not always reproducible. Many materials, such as the dimeric alkaloids used in this composite, occur at nearly the same location; it is not possible to distinguish between them in this system.

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Assay for Iodochlorhydroxyquin in Iodochlorhydroxyquin Ointment

By JACK COHEN† and ELMER KLUCHESKY

The U.S.P. assay for iodochlorhydroxyquin in iodochlorhydroxyquin ointment was found, in our laboratories, to be unworkable with either hydrocarbon or hydrophilic ointment bases. A colorimetric method was developed which is rapid, reproducible, accurate, and applicable to various ointment formulations. It is believed that this procedure is superior to the official assay presently in use.

THE U.S.P. XVI (1) assay for iodochlorhydroxyquin in ointment formulations involves the precipitation of iodochlorhydroxyquin as a copper chelate from an acetone extract of the ointment and subsequent gravimetric determination by weighing the precipitate.

Attempts were made without success to utilize this assay method in the determination of iodochlorhydroxyquin in two ointment formulations —one an oil-in-water emulsion and the other a hydrocarbon base. The ointment base ingredients do not remain in solution during precipitation of the iodochlorhydroxyquin complex thus seriously contaminating the precipitate on the filter.

Therefore, a method was sought for the assay of iodochlorhydroxyquin in ointment formulations which would circumvent these difficulties and, at the same time, find application to a variety of different types of ointment bases. A colorimetric assay based on the reaction described by Haskins and Luttermoser (2) was developed and found to be applicable to both of the formulations studied. The method depends upon the formation of a colored complex between iodochlorhydroxyquin and ferric ion at a pH between 1.0 and 2.0. Table I demonstrates the application of the method to quantitatively prepared standard samples.

TABLE I.—RECOVERIES OF IODOCHLORHYDROXYQUIN FROM STANDARDS

Hydrophilic Base, %	Hydrocarbon Base, %
100.0	99.4
98.5	99.1
101.5	98.8
101.0	101.0

EXPERIMENTAL

Apparatus and Reagents.—A Beckman DU spectrophotometer with tungsten light source and 1-cm. cells; acetone, reagent grade; methyl cellosolve, reagent grade; and iron reagent (prepare by adding 1 Gm. of ferric chloride, reagent grade, and 1 ml. concentrated hydrochloric acid to a 1-L. volumetric flask and diluting to volume with water) were utilized in this study.

Procedure.—Using a hypodermic syringe with a needle large enough to easily accommodate the ointment, add approximately 3 Gm. of ointment to a tared 100-ml. volumetric flask and weigh to determine the sample weight. Add 50 ml. of acetone to the contents of the flask and heat the mixture on a steam bath with occasional swirling until the ointment melts. Stopper the flask, shake vigorously for a short while, and allow to cool to room temperature. Dilute to volume with acetone and mix thoroughly. Add a 3-ml. aliquot to a 25-ml. volumetric flask, using a pipet with a cotton pledget over the tip. Evaporate the acetone from the flask on a steam bath and chill the residue briefly in an ice bath until it solidifies. To the residue add 20 ml. of methyl cellosolve and swirl to disperse the solid material (do not shake vigorously). Add 2 ml. of iron reagent, dilute to volume with methyl cellosolve, and mix thoroughly. Prepare a reagent blank solution by diluting 2 ml. of iron reagent to 25 ml. with methyl cellosolve. Insert a cotton swab on the end of a glass rod into the solution in the neck of the flask and withdraw it to remove most of the oil globules from the surface of the solution. Fill a 1-cm. Pyrex cell using a 4-ml. pipet with a cotton pledget over its tip. The solution at this point must be clear. Determine the absorbance of the solution against the reagent blank at 650 mμ on the spectrophotometer.

Calculations.—Calculate the amount of iodochlorhydroxyquin in the sample by

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mg. iodochlorhydroxyquin per Gm.

$$\text{of ointment} = \frac{A}{K \times \text{Gm. of sample}}$$

where A = absorbance at 650 mμ, and K = absorbance index in terms of mg. of drug per 100 ml. of sample solution.

Absorbance Index.—Prepare three standard solutions of iodochlorhydroxyquin reference standard in methyl cellosolve to contain approximately 30, 50, and 70 mg. per 100 ml. Develop the color for each standard as follows. Add a 3-ml. aliquot to a 25-ml. volumetric flask and add 17 ml. of methyl cellosolve. Add 2 ml. of the iron reagent, dilute to volume with methyl cellosolve, and mix thoroughly. Prepare a reagent blank by diluting 2 ml. of the iron reagent to 25 ml. with methyl cellosolve. Determine the absorbance at 650 mμ using the 1-cm. cells and the reagent blank solution in the reference cell. Calculate the absorbance index K by the equation $K = A/c$, where c is the concentration of standard in mg. per 100 ml. and A is the observed absorbance for the corresponding colored solution. Table II shows the results for three such standards using the Beckman DU spectrophotometer.

TABLE II.—DETERMINATION OF ABSORBANCE INDEX ($K = A/c$)

Concn. c, mg. per 100 ml.	Absorbance A	Absorbance Index K
34.8	0.218	0.00626
52.0	0.320	0.00615
69.5	0.427	0.00614

CONCLUSIONS

The U.S.P. method for iodochlorhydroxyquin in ointment preparations presents many difficulties. It is practically impossible to obtain a precipitate free of ointment base materials from an acetone extract of most ointment preparations.

The method described in this paper offers several advantages over the official method in that it is simple, rapid, reproducible, and accurate. The color formed by the reaction of ferric ion and iodochlorhydroxyquin in methyl cellosolve has been shown to be stable over a period of at least 1.5 hours. The application of the assay to two different types of ointment bases was demonstrated.

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Technical Articles

Preparation of Parenteral Dispersions

By THOMAS J. MACEK

Parenteral dispersions of medicinal products may take the form of parenteral colloids, parenteral emulsions, or parenteral suspensions. The paper defines these categories and discusses problems of manufacture and stability and illustrates with practical examples.

THE TERM "dispersion" is very general and may have several meanings. A simple solution of a salt or sugar in water can be considered an aqueous dispersion. As such, it occupies a position at one end of a scale describing various states of matter. Heterogeneous admixtures of a liquid and relatively large fragments of another immiscible liquid or solid, on the other hand, are described as emulsions or suspensions and occupy a place at the opposite end of that scale. The state of matter between these two extremes, and more particularly when the dispersed phase consists of particles between 1 and 100 mμ in size is that intermediate range called the "colloidal state." This region frequently is subdivided further by the term "colloidal solution" or "sol" as in the case of a solution of gelatin or of silver iodide, and colloidal dispersions, as in the case of colloidal gold having solids suspended in the submicron form. The term "hydrosol" is employed when the solvent is water, and the term "aerosol" refers to a colloidal dispersion in air or another gas.

Actually, the line of demarcation between a true solution and the colloidal state is not really very sharp. Solids dissolve in a liquid such as water when there is complete or near complete intermingling of solute and solvent molecules or ions. The ionic bonds of salts such as exist between Na^+ and Cl^- must be fractured in the process of dissolving, the energy being derived from the polarity of the solvent. At best, solute to solute and solvent to solvent bonds have to be broken and new solute to solvent bonds formed. If the forces—such as

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mg. iodochlorhydroxyquin per Gm.

$$\text{of ointment} = \frac{A}{K \times \text{Gm. of sample}}$$

where A = absorbance at 650 mμ, and K = absorbance index in terms of mg. of drug per 100 ml. of sample solution.

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hydrogen bonding—between these new associations are great, solubility is favored. In that special situation where these forces are equal or cancel previously existing forces between individual particles of solute and individual particles of solvent, there is a free intermingling and we have a case of an ideal or perfect solution. On the other hand, the forces between the dissimilar particles much more frequently are not as great as the forces between the similar individual components; hence solubility will not be favored, but dispersion may occur. The dispersion may assume colloidal or suspension proportions, sometimes followed by precipitation or flocculation or both.

In accordance with this concept, the terms "hydrophilic" and "hydrophobic" are used to describe the tendency of a surface, a particle, or a functional group to establish bonds of association with the solvent, water. In colloidal systems, stable colloids are always referred to as lyophilic or hydrophilic. Unstable colloids are always lyophobic or hydrophobic. They represent those that are readily flocculated by small amounts of electrolytes.

Some examples will serve to illustrate. Albumin and other proteins dissolve in water, forming hydrophilic sols. Polyvinylpyrrolidone, dextran, the carbohydrate gums, and other synthetic gums behave similarly. In spite of the large size of the protein or gum molecules, they form remarkably stable colloidal solutions. This is because of their state of hydration (the so-called watery envelope) and of their electrostatic charge. To precipitate a hydrophilic sol, one must first remove the charge. This usually is accomplished by adjusting the pH to the isoelectric point at which there is electrical neutrality. Then the watery envelope is removed by dehydration, as with alcohol or a dehydrating salt, such as ammonium sulfate. The protein or gum precipitates under these rather special conditions.

Hydrophobic sols behave quite differently. If an insoluble substance is ground long enough and fine enough in water, a hydrophobic sol may form. This colloidal state will not be very stable, however. The particles, continually in motion, bombard each other, energy is reduced in the process, and there is a great tendency toward coalescence and flocculation. Hydrophobic sols are stabilized by reducing interfacial tension which changes the energy at the particle surface. The process of transforming an insoluble substance into the colloidal state frequently is referred to as "peptization." However, even this colloidal state is easily broken by the simple addition of an electrolyte.

When the extent of the stabilization by reduction of surface forces is great enough, a hydrophobic material can be rendered seemingly water-soluble. This process is called "solubilization," or solubilization through micelle formation. It differs from hydrotropy, another solubilization process which is distinguished by the absence of colloidal material. In the latter case, solubilization is accomplished through the use of aqueous solutions of salts or organic ions, the so-called "salting in" effect, or through the use of water-diluted organic solvents or mixtures of solvents. Emulsification is distinguished from both solubilization and hydrotropy principally by the size of the dispersed phase. An emulsion generally also is opaque, whereas the other phenomena produce solutions which are transparent, at least to the naked eye. Furthermore, an emulsion comprises a dispersion of one immiscible liquid in another. When the process describes solids instead of liquids, the system is a suspension.

Over the years, numerous parenteral formulations have been developed for medicinal use which span the entire range of these different states of dispersion. Much could be said about the properties of surface-active agents, about the hydrocolloids, and about techniques and theories involved in the dispersion and emulsification processes. However, inasmuch as industrial pharmacists constantly are faced with practical problems of development and manufacture, this paper concerns itself with some practical considerations pertaining to parenteral colloids, emulsions, and suspensions. It is hoped that observations and experiences in the development of these dispersion products may prove interesting while illustrating principles and problems which are encountered when working in this field.

MATERIALS AND FACILITIES

The choice of active form of the drug for the preparation of parenteral dispersions is limited largely by pharmacological considerations. Therefore, in most cases the industrial research pharmacist is confronted with a form of a drug that is active biologically with which he must prepare safe and acceptable parenteral products. Quite often, this may be the only active form of the drug known at the time. In some cases, only insoluble forms of the drug possess desirable properties such as prolongation of activity, stability, lack of irritation, etc.

Such was the case at first with the insoluble derivatives of the corticosteroids. Several years elapsed before stable, biologically active, and completely water-soluble derivatives of the adrenocortical steroids were discovered. As a general rule, when biological properties are equal, it is far more desirable and economical to search out water-soluble forms of new drugs for parenteral administration than to attempt the more difficult formulation of physically less stable parenteral dispersions. In the case of methyldopa,[1] exactly this course was pursued, resulting in the development of the water-soluble and biologically active ethyl ester hydrochloride derivative specifically for parenteral use. Other well-known examples of this approach have occurred among the antibiotics. The sodium methanesulfonate of colistin provides a water-soluble parenteral. The ethanol-ammonium-magnesium complex of oxytetracycline is soluble in a propylene glycol-water vehicle employed for injection. The ethyl succinate of erythromycin has been dissolved in a polyethylene glycol vehicle for intramuscular administration. A 10% solution of the sodium succinate derivative of chloramphenicol proved useful for both intravenous and intramuscular injection. Similar examples are found among other classes of therapeutic compounds.

On the other hand, the natural oil form of vitamin K_1 proved much superior therapeutically to syn-

[1] Marketed as Aldomet by Merck and Co., Rahway, N. J.

thetic analogs of K and had to be dealt with even though water-insoluble. For reasons of stability, the insoluble salts of penicillin had to be employed to formulate ready-to-use aqueous suspensions of this antibiotic. The insoluble dibenzylethylenediamine salt of penicillin had to be used to achieve the sought-after prolongation of blood levels. And so the problems of preparing parenteral dispersions persist.

Agents which are employed to prepare and stabilize parenteral dispersions have to meet at least three principle criteria. The first is that they must be safe. This requires that they be nontoxic, nonantigenic, nonpyrogenic, nonirritating, and nonhemolytic. The second is that they must be potent dispersants and/or stabilizers so that only small amounts are required. Third, the agents should be stable, especially upon prolonged storage, and preferably under the conditions of higher temperatures which one would like to employ for positive sterilization. One might add a fourth criteria—namely, known acceptability to the federal Food and Drug Administration. Otherwise, the burden of proof of safety, which today if not attainable without considerable expenditure of time and money, rests entirely upon the user. These are very rigid specifications and rule out many agents that are perfectly satisfactory for oral use in pharmaceuticals and even in foods. A partial list of agents which have been employed in commercial parenteral products is shown in Table I.

The preparation of parenteral dispersions often presents difficult or unusual problems of manufacture because of the requirements for sterility and freedom from pyrogen contamination. In this regard, the efficiency of dispersing and mixing equipment sometimes must be comprised to achieve completely aseptic operation. The alternative is to reconstruct such equipment to withstand heat sterilizing conditions, also a job not easily accomplished. Totally enclosed systems have had to be designed for large-scale aseptic production operations. In such instances assurance was needed to show that the dispersions could be adequately mixed and kept in a state of uniform suspension throughout all preparative, transfer, and subdividing operations. This has been a task to engage the best thinking and ingenuity of both pharmacists and engineers in many industrial plants. Apart from the facilities and techniques for sterile formulation, the development and production of parenteral dispersions would not have been possible without considerable attention to the physical properties of the starting materials, particularly in dealing with suspensions.

In this regard the first gram of cortisone acetate ever produced was processed by aseptic milling with aqueous vehicle employing a glass bottle and glass beads. Milling continued until 90% of the particles were smaller than 10 μ. Although the procedures changed drastically as the scale of operation increased, the requirement for the particle size was retained and, indeed, established a standard for most of the corticosteroid suspensions that followed. Today cortisone acetate and other corticosteroids for parenteral use are crystallized using methods and facilities which produce a sterile solid having a particle size smaller than 10 μ. In addition, the crystallization of cortisone acetate is conducted in a way which produces that polymorphic form which experience has shown to be stable upon prolonged storage in aqueous suspensions. Such starting material requires no subsequent milling. It is simply suspended in a sterile aqueous vehicle by a process which lends itself readily to large-scale operation under strictly aseptic conditions.

TABLE I.—MATERIALS USED IN PARENTERAL DISPERSIONS

Surfactants
Pluronic F-68 (polyethylene-polypropylene nonionic)
Polysorbate 80 U.S.P.[a]
Polyoxyethylene sorbitan monolaurate
Emulphor EL-620
Sorbitan trioleate[b]
Lecithin
Hydrocolloids
Sodium carboxymethylcellulose
Polyvinylpyrrolidone
Gelatin (nonantigenic)
Methylcellulose
Other
Polyethylene glycol 300
Propylene glycol
Sorbitol
Silicone antifoam
Aluminum monostearate

[a] Polyoxyethylene 20 sorbitan monooleate, marketed as Tween 80 by Atlas Powder Co., Wilmington, Del. [b] Marketed as Span 85 by Atlas Powder Co., Wilmington, Del.

The manufacture of other parenteral suspensions presents somewhat different problems. Nonetheless, detailed attention is required to specifications of crystal form and particle size in order to achieve physical and therapeutic uniformity in the final parenteral dispersions.

PARENTERAL COLLOIDS

The area of biological products provides numerous examples of parenteral dispersions in the colloidal state. These include serum albumin, fibrinogen, various immune globulins, human fibrinolysin, insulin, corticotropin, and other hydrophilic protein or peptide products derived from human blood and natural sources.

Several parenteral solutions of iron are actually colloidal dispersions of complexes of iron with carbohydrate materials. This category includes an iron-dextran complex,[2] a high molecular weight iron-carbohydrate,[3] and a saccharated iron oxide.[4]

Perhaps of greater interest, however, are those colloidal dispersions prepared by micellular solubilization involving smaller organic molecules that are essentially hydrophobic. Parenteral products in this category include a product containing water-solubilized vitamin A,[5] an aqueous dispersion of a synthetic vitamin K,[6] and an aqueous colloidal

[2] Marketed as Imferon by Lakeside Laboratories., Inc., Milwaukee, Wis.
[3] Marketed as Astrafer I. V. by Astra Pharmaceutical Products, Inc., Worcester, Mass.
[4] Marketed as Proferrin by Merck Sharp and Dohme, West Point, Pa.
[5] Marketed as Aquasol A by U. S. Vitamin and Pharmaceutical Corp., New York, N. Y.
[6] Marketed as Konakion by Hoffmann-LaRoche, Inc., Nutley, N. J.

solution of vitamin K_1[7] made using Emulphor EL-620 as solubilizing surfactant.

The theory of solubilization by micelle formation was first postulated in 1913 by McBain (1). It described the phenomenon wherein particles of a colloidal substance such as potassium stearate functioned to incorporate the insoluble materials within themselves. In the process the original solid or oil particles disappeared into the organized structure of the stable colloid. This process today has been extended to include a wide variety of surface-active agents, some of which are acceptable for parenteral dispersions.

To be effective the surfactants themselves must be soluble in water and form the proper micellular environment for solubilization. The concentration at which micelle formation begins to occur is known as the critical micelle concentration. This can be measured in various ways and has been observed to decrease as chain length of surfactant increases, regardless of its basic organic structure. Sometimes the surface-active solubilizer may have other effects which enhance its activity, such as cosolvency and specific complex formation. However, in spite of many excellent studies on solubilization which have brought about a better understanding of some of the mechanisms involved, it still is necessary to conduct empirical research to arrive at practical answers in most cases.

Some generalizations are available, however, and are worth noting. In 1949, Griffin (2) devised a hydrophile-lipophile balance (HLB) system for classifying surfactants which is helpful in selecting solubilizers. Moore and Bell (3) later listed some useful observations based upon the studies of a number of workers which are helpful in laboratory problems of solubilization. For example, it has been observed that in a homologous series of compounds the amount of solubilizer needed increases directly with the polarity, cyclization, and unsaturation of the hydrophobe and inversely with chain length. The addition of inorganic salts to a solubilized system increases the amount of nonpolar or hydrocarbon-like substances solubilized, but decreases the amount of polar substances solubilized. The more lipophilic nonionic surfactants favor maximum solubilization of low polar substances, and the more hydrophilic nonionic surfactants favor solubilization of the more polar hydrophobes.

From a formulator's viewpoint, it is often found that a mixture of two or more surfactants proves better for solubilizing a compound than does one alone. In addition, a co-solvent sometimes also helps to attain a clear solubilized solution. Once a solubilizer or mixture of solubilizers shows promise, the effect should be studied over a range of ratios of solubilizer and solubilizate. And finally, a mixture of water, solubilizer, and hydrophobic solubilizate often has to be heated to obtain a solution. The optimum heating time may vary with different systems. In some cases, mixtures may show a negative solubility. In other words, their solutions may be cloudy at higher temperatures and clear at room temperature. Clarification may develop slowly in these instances and the solution therefore should be allowed to stand. Slightly opalescent solutions sometimes may be made crystal clear by rapid cooling.

Even though the list of parenteral products made by solubilization using surfactants is still small, this area of formulation offers interesting possibilities. It not only provides a means for preparing aqueous parenteral forms of hydrophobic drugs, but also provides the opportunity for stabilization of some sensitive drugs in parenteral products as well. In this regard, Riegelman (4) observed that micelles of cationic or anionic surfactants shielded certain esters against hydrolysis.

In a 5% sodium lauryl sulfate solution, the half-life of benzocaine against base-catalyzed hydrolysis was extended some eighteenfold. This is a very profound change and is worth noting. Although sodium lauryl sulfate may be unsuitable for parenteral use in these quantities, other surfactants need to be studied. The ampholyte, Emulphor EL-620, by comparison is much less toxic, and also nonhemolytic in rather substantial concentrations.

PARENTERAL EMULSIONS

Until now only two commercially-available parenteral emulsions have enjoyed extensive medical use. Both of these were specifically for intravenous administration. However, parenteral emulsions of fats, either alone or supplemented with amino acids or vitamins, attracted considerable research and clinical interest some time ago and occupied the attention of pharmacists for several years.

An emulsion of a natural vitamin K_1 in an aqueous vehicle made with a purified form of lecithin as emulsifier is described in the U.S.P. as sterile phytonadione emulsion.[8] Considerable care is taken during manufacture and testing of this parenteral to assure that all of the emulsified oil droplets conform to a rigid particle size specification in the range of 1 to 5 μ. This state of subdivision is achieved by an emulsification process involving the use of an intermediary solvent. The emulsion is sterilized by autoclaving while in bulk; it is then filtered and subdivided aseptically into the final ampul containers.

The second product is a parenteral fat emulsion[9] containing 15% cottonseed oil and 4% dextrose, with 1.2% lecithin and 0.3% of an oxyethylene-oxypropylene polymer as emulsifying agents. The product is administered in volumes of 250 to 500 ml. by intravenous infusion to supply dietary fat in debilitating diseases, post surgery, etc. Most of the dispersed droplets in this product are less than 1 μ in size and none can be greater than 5 μ. The product presumably is sterilized in the final container by autoclaving. Like sterile phytonadione emulsion, it must be protected against freezing.

Inasmuch as parenteral emulsions are intended for intravenous administration, absolute assurance of sterility is required, and sterilization in the final container by autoclaving is virtually essential.

[7] Marketed as Aquamephyton by Merck Sharp and Dohme, West Point, Pa.

[8] Marketed as Mephyton by Merck Sharp and Dohme, West Point, Pa.

[9] Marketed as Lipomul I. V. by The Upjohn Co., Kalamazoo, Mich.

Elevated temperature, however, tends to cause increased coalescence of fat globules; hence, parenteral emulsions must be designed to withstand heat sterilization. In this connection, nonantigenic gelatin has been explored for use both as an emulsifier and emulsion stabilizer for intravenous products. Unfortunately, solutions of gelatin hydrolyze upon autoclaving, and this change can result in alterations in emulsion stability. Small concentrations of methylcellulose appear to stabilize fat emulsions against separation or creaming, but one encounters a problem during autoclaving because of the insolubility of methylcellulose at elevated temperatures. Dextran also has been examined as an emulsion stabilizer.

The stability of fat emulsions is no small problem. Apart from the potential development of rancidity of the oil phase, fat emulsions are subject to adverse physical changes. Excessive shaking of parenteral fat emulsions has been observed to accelerate the rate of separation ("creaming") and to cause a greater tendency toward particle coalescence. This change probably is caused by rupture of the film of emulsifier about the particles of dispersed oil. It has been also observed that bottles half full of fat emulsion "creamed" at a faster rate upon prolonged storage than full bottles. Such change was thought to be due to decomposition of the film of emulsifying agent by oxidation.

It is apparent, then, that the preparation of parenteral emulsions is troublesome. It is made more difficult by rigid requirements regarding particle size for reasons of safety, without much latitude in the choice of emulsifiers and stabilizers and stringent requirements for stability upon exposure to both high and low temperatures.

PARENTERAL SUSPENSIONS

One of the earliest commercial parenteral suspensions was sterile bismuth subsalicylate suspension in oil. The product still is described by U.S.P. monograph and is employed as an antiprotozoal and antisyphilitic agent. Among other early parenteral suspensions were those of the estrogens, androgens, and progestational hormones, some of which still are very valuable therapeutic agents.

However, it was the discovery of the adrenocorticol stercids, of penicillin, and the broad spectrum antibiotics that gave renewed impetus to the development of new compositions and technology for the preparation of parenteral suspensions.

The method employed for preparing the first quantities of cortisone acetate was mentioned previously. Glass bead milling to reduce particle size was conducted in a portion of the vehicle containing polysorbate 80 as surfactant to facilitate wetting of the water-insoluble, hydrophobic crystals of the steroid. The wetting agent was effective in this case because it favored the replacement of the solid-air interface by a solid-liquid interface. This is the property expected of a good dispersing agent. However, this alone was insufficient to produce a physically stable suspension. Hence, there was a need for a compatible dispersion stabilizer, protective colloid, or agent to bridge the gap between the continuous phase of the aqueous vehicle and the essentially hydrophobic particles which it enveloped. For this purpose sodium carboxymethylcellulose was employed. This is a safe parenterally acceptable hydrocolloid which had an affinity for the continuous phase. It could be employed effectively in concentrations which produced only a small change in viscosity; it was stable and could be sterilized by autoclaving. In the presence of sodium carboxymethylcellulose, to a large extent the dispersed particles of steroid lost many of the properties which normally identified them as hydrophobes—namely, surface charge, water repellency, and tendency toward agglomeration. Instead they assumed some of the properties of the protective colloid and of the continuous phase. Thus, the suspension was stabilized, settling was reduced, and resuspension by shaking was readily accomplished. Sodium chloride was added to adjust isotonicity. Benzyl alcohol was employed as a preservative. This example illustrates one of the fundamental ways available to the pharmacist for preparing suitable suspensions of a hydrophobe for parenteral use—namely, dispersion using a wetting agent in conjunction with a compatible protective colloid.

A brief digression for a moment will serve to highlight another potential problem. During the period that parenteral suspensions of cortisone acetate were made by bead milling, no problems with polymorphic changes were encountered. It is apparent today that phase changes were being experienced, inasmuch as the starting solid was later shown to be a physically unstable variety in contact with water. However, if a change occurred, this happened during the course of the extended milling cycle and without evidence of significant changes in particle size. Difficulties with polymorphism were encountered when dry, premilled solid of the wrong crystal form was rapidly suspended in the aqueous vehicle by mechanical agitation and the resulting suspensions were allowed to stand undisturbed. The change to a more stable crystal form invariably was associated with crystal growth, the intermingling of these crystals, and the formation of a cake or lumpy suspension. Usually these changes occurred in the final vials so that little could be done to recover the product. The possibility of polymorphic change, therefore, should be anticipated in many parenteral suspensions of new compounds, especially of the steroid type.

Recently, with some steroids, particularly the highly water-insoluble tertiary-butylacetate esters of hydrocortisone and prednisolone, it was possible to replace sodium carboxymethyl cellulose in the aqueous vehicle with sorbitol. This additive functioned as a suspension aid by increasing the density of the aqueous vehicle rather than as a protective colloid. The rate of settling of the suspended solid was readily decreased as the density of the aqueous phase increased by addition of graded increments of sorbitol.

The preparation of aqueous parenteral suspensions of procaine penicillin both in the ready-made and dry-mix forms presented some very different and interesting dispersion problems. Where concentration of suspended solids did not exceed 5% in the case of the corticosteroids, the problem now was one of preparing essentially fluid, syringeable, and injectable suspensions containing up to about 35% solids. Without effective dispersing agents, it

was very difficult even to wet this much solid adequately with the limited volume of water. Surfactants such as lecithin and Pluronic F-68, which proved very effective for purposes of wetting, produced highly dispersed suspension systems exhibiting extremely poor physical stability. Such systems settled slowly but ultimately sedimented to a very dense cake which was exceedingly difficult to resuspend. When viewed under the microscope, disperse suspensions of this type were seen to consist of individual particles, each in random and haphazard motion in relation to others having no apparent association between themselves. The dispersion could be likened to a suspension of sea sand in a bottle of water. Each particle was free to move in any direction at any speed even after colliding with another. When gravity sedimentation occurred, the volume occupied by the solid was small. The dense cake which formed, however, was resuspended again only by peeling a thin layer of particles off the top, one after another, until finally all solid was resuspended.

With procaine penicillin dispersions, it was found that not enough protective colloid could be added to improve adequately the sedimentation properties without also significantly altering their viscosity, flow, syringeability, and other physical properties, usually in an undesirable way. This is not to say that hydrocolloids, like sodium carboxymethylcellulose or gelatin, did not produce a favorable result in aqueous suspensions of procaine penicillin. If the products had been for oral use where high viscosity was not of great importance, they probably would have been quite acceptable. Indeed, the addition of gums is a common way of stabilizing oral suspensions. However, in the parenteral suspension the properties sought included a high degree of product fluidity, little or no foaming, quick restoration with water when in the dry form, a rapid break from the surface of the siliconed vial, a thin suspension for easy syringeability, and a product with good injectability through small bore hypodermic needles. Such properties could not be achieved readily in suspensions of high solids content by the classic method of using a protective colloid alone in conjunction with a wetting agent. Some other method for suspension stabilization had to be found. In this connection partial flocculation by the addition of selected inorganic ions proved to be highly successful.

Flocculation of the dispersed solid was achieved in increasing magnitude by monovalent, divalent, and trivalent ions, respectively. Indeed, flocculation of procaine penicillin dispersions by the addition of even very small amounts of trivalent aluminum, as the chloride, would occur to such an extent that the suspensions acquired an unsightly, nonuniform appearance. However, such suspensions could always be resuspended by gentle shaking. In practice, a partially flocculated dispersion made with monosodium citrate proved most practical. A small amount of the protective colloid, or the use of sorbitol to increase vehicle density, also proved of benefit.

According to Michaels and Bolger (5), in the partially flocculated system, the floc, or the associated colony of a number of particles, becomes the basic flow unit in low shear processes such as gravity sedimentation. These flocs can be regarded as rigid spheres which tend to cluster together in weak aggregates. The aggregates grow by collision, are broken down by low shear forces such as gentle shaking or by flow through a small orifice of a hypodermic syringe or needle, yet they are able to reform easily the extended networks which give the suspension its structural properties with virtually no increase in viscosity. The partially flocculated suspension will settle rapidly, but to a high sedimentation volume and is easily resuspendable. Most important from our standpoint, they can be produced easily under sterile conditions, using materials which are fully acceptable for parenteral formulation.

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Wood Products, Corncob, and Cellulose as Tablet Disintegrating Agents

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A comparative study of certain powdered wood products, corncob, and cellulose has been made for their tablet disintegrating properties. A brief description for preparing the powdered disintegrating agents from the natural products is given. The various disintegrating agents were incorporated into lactose granulations, compressed, and subsequently tested for tablet disintegration time. Powdered corncob was found to be superior to starch and the wood products as a tablet disintegrating agent. The results of the disintegrating action of powdered corncob proved to be reproducible. Powdered redwood bark was as good as starch but not as effective as powdered corncob. The three physically distinctive layers of corncob—chaff, woody substance, and pith—were separated and incorporated in lactose tablets as disintegrating agents. Significant differences were seen among the disintegration times produced by the three layers of corncob. The pith showed superiority over the chaff and woody portion. A series of tests was conducted to study the separate and combination effects of cellulose and starch—the two main constituents of corncob—on tablet disintegration. Cellulose was found to be a better tablet disintegrating agent than starch. The combination of cellulose and starch indicated better results than the separate action of either agent alone. The possible mechanism of action for tablet disintegration is discussed.

THE IMPORTANCE of proper tablet disintegration cannot be overemphasized. To be effective, it is necessary for the tablet to break apart within a desirable period of time so that the medication may be absorbed. To accomplish this, a substance is added to the tablet granulation that will react when brought into contact with fluids and cause the tablet to break apart. Such a substance is termed a disintegrating agent. Disintegrating agents generally fall into three classes, according to their mechanism of action: (*a*) agents which react with moisture to constitute a foam, (*b*) effervescent substances which react with moisture to form a gas, (*c*) substances which react with moisture and swell. Those of the "absorb moisture and swell" type are by far the most commonly employed in research and industry today (1–8), and the present study is confined to this group.

Disintegration time is a function of many factors (4): (*a*) the particular characteristics of the tablet machine, (*b*) the basic formula used in preparing the granules, (*c*) the size, shape, weight, hardness, and age of the tablet, (*d*) the particle size of the medicament, and (*e*) the method of measuring tablet disintegration.

In addition to these variables Ward and Trachtenberg have included (9): (*f*) the moisture content of the granules, (*g*) the particle size of the granules, and (*h*) the particle size of the disintegrants.

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In this study each of these factors was held constant or minimized to reduce experimental error.

Billups and Cooper (2) introduced wood flour and powdered wood bark as possible tablet disintegrating agents. In an attempt to continue their work, this research was originally based on testing a large number of wood products for their tablet disintegrating effects. It was during this phase of the research that powdered corncob, one of the agents included in the preliminary study, produced significantly rapid tablet disintegration times. Because of the superior results obtained with powdered corncob, intensive study was conducted with this substance.

This paper presents the results of a comparative study of several wood products, powdered corncob, and cellulose for their potential tablet disintegrating properties. These products were compared with cornstarch, the classical and still one of the best tablet disintegrating agents known. The objectives of the study were to discover new products with superior tablet disintegrating ability and to propose a mechanism of action for the disintegrating agents tested.

EXPERIMENTAL

Selection of Disintegrating Agents.—The selection of various disintegrating agents and the reason for their selection was of primary importance.

Cornstarch was chosen because of its popularity and extensive use. Many authorities still consider this classical agent as the best tablet disintegrating agent in use today (2, 10, 11). A U.S.P. grade of cornstarch was employed in this study.

Powdered redwood bark was included as a new

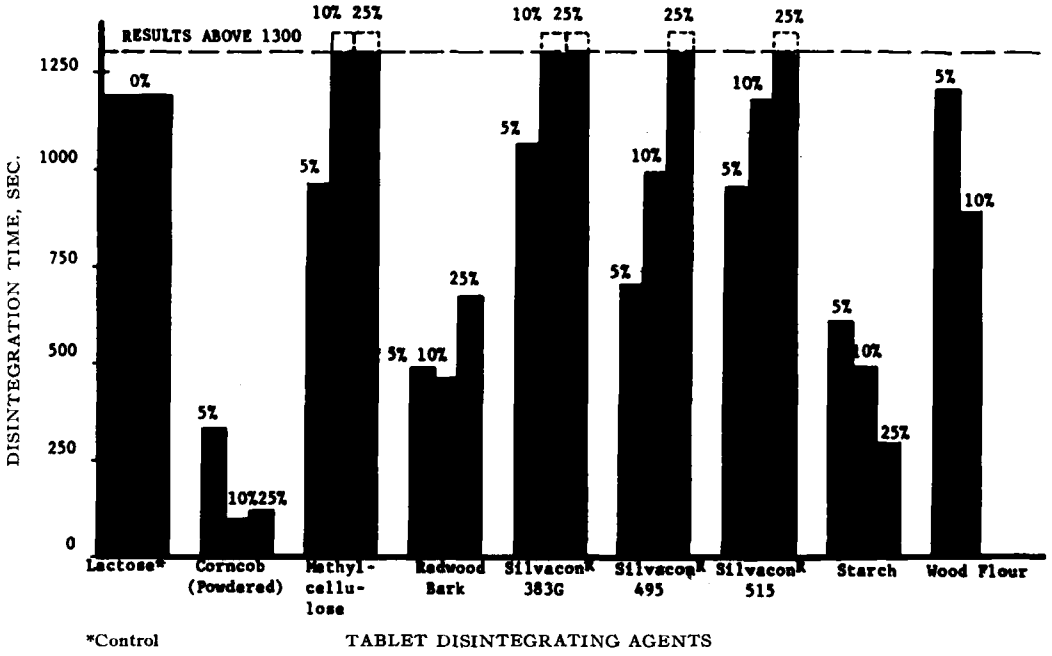

Fig. 1.—Disintegration times of tablets containing various disintegrating agents.

agent to be tested for tablet disintegrating properties. This agent is the powdered bark from the coniferous timber tree (*Sequoia sempervireus*) of California, found only on the Coast Range. The powdered bark was prepared by passing bark specimens, dried 2 months at 80° F., through a Wiley mill until the powder was sufficiently fine to pass through a No. 40 sieve.

Wood flour (2) was used in this study to compare with other wood products. Wood flour is the powdered inner wood from Douglas fir (*Pseudotsuga taxifolia*), a tall evergreen tree of the western United States, often known in the lumber trade as red fir or Oregon pine. The wood flour was obtained locally from the Forest Products Research Laboratories, Corvallis, Oreg.

Silvacon[1] is powdered bark from Douglas fir species. The three basic types of Silvacon are pliable, spongy flakes 383-G, tough needle-like fibers 515, and fine amorphous powders 495. Billups and Cooper used only Silvacon 383-G in their study. All three forms of Silvacon were used in this research. Silvacon products are natural plastics containing the plasticizers, cellulose, and resins found in Douglas fir bark (12).

Methylcellulose was included for comparison with the wood products. Methocel,[2] having a viscosity of 4000 cps. was used.

Powdered corncob, *Zea mays* Linné (Fam. *Gramineae*), was chosen because of its similarity to the other woody products. Corncob, considered a waste product of corn, is used only in small quantities to feed cattle, fertilize land, act as a fuel in remote farming areas, and form chemical by products. The corncob used in this study was obtained locally from the Department of Farm Crops, Oregon State University. A 50-pound randomized sample of corncob was dried for 2 months at 80° F. and powdered with a Wiley mill. Both field corncob and popcorncob were used in the preliminary tests, and since there was an indication that field corncob was slightly better as a disintegrating agent than popcorncob, the former was used throughout this study.

Lactose, a substance with no apparent tablet disintegrating property, was used as the control. Lactose is regarded as a good diluent or inert base for many tablet preparations and was used as a diluent throughout this work.

Procedure for Manufacture of Tablets.—The method of wet granulation was used to manufacture all of the tablets in this study. Tablets were prepared containing lactose as the diluent, 1% magnesium stearate as the lubricant, and syrup U.S.P. as the granulating agent. The finished granulation was separated into "fines" or particles smaller than No. 40 mesh[3] and "granules" or larger particles ranging between No. 14 and No. 40 mesh. According to the amount of disintegrating agent used, an appropriate quantity of "fines" was replaced in the granulation so that all tablets were derived from mixes containing similar proportions of "fines" and "granular" material. A ratio of 30% "fines" to 70% "granules" was used throughout this study. Since the disintegrating agents were added as "fines" to the finished granulation, they were never in contact with moisture during tablet manufacture.

The tablets were compressed on a Stokes model B-2 rotary tablet machine using $^3/_8$-in. diam. punches and dies. Each tablet batch consisted of 500 tablets. An average theoretical weight of 500 ± 10 mg. was maintained using an exact weight shadow-

[1] Marketed by Weyerhauser Timber Co.
[2] Marketed by Dow Chemical Co.
[3] All of the sieves employed in this study compiled with the standards specified in the U.S.P.

graph calibrated periodically with a torsion balance. This average tablet weight was obtained by selecting five samples from each 500-tablet batch. Each sample taken consisted of ten tablets.

Measurement of Hardness and Disintegration Time.—Tablet hardness was determined on a Strong-Cobb hardness tester for ten tablets randomly selected from each tablet batch. Preliminary studies indicated that a tablet hardness of 10 ± 1 Strong-Cobb units gave the least variation in tablet disintegration time and was used unless otherwise specified. Tablets in this hardness range were found to be more resistant to breaking and chipping when subjected to friability tests simulating the most extreme handling conditions. Finally, a hard tablet was felt to reveal better the disintegra ing ab lity of the agents selected for study.

The disintegration tests were conducted on a Gershberg-Stoll (13) type disintegration apparatus in accordance with the official U.S.P. method (14). The reported disintegration time for each batch of tablets represents an average time for 12 tablets. Distilled water was used as the disintegration medium.

RESULTS AND DISCUSSION

The research was divided into three parts: the comparative disintegration tests of wood products and other tablet disintegrating agents, the disintegrating effects of powdered corncob, and the role of cellulose in the disintegrating activity of powdered corncob.

Comparative Disintegration Tests.—The first phase of this research concerned a comparative study of the wood products and other agents for their tablet disintegrating ability. Accordingly, each of the selected agents was incorporated into a standardized lactose granulation in 5, 10, and 25% concentrations. The tablets were compressed and tested for disintegration time immediately after manufacture. The results (summarized in Fig. 1) indicate a significant variation in disintegration time among the various agents. Powdered corncob had the shortest disintegration time of all agents tested. All concentrations of corncob gave better results than starch. A 10% concentration of powdered corncob was the best disintegrating agent of the entire test series. Concentrations of 10% wood flour, 5 and 10% Silvacon and 5, 10, and 25% redwood bark gave improved disintegration times compared to the control. Methylcellulose and the Silvacon products in higher percentages gave longer disintegration times than the control. The use of these substances as disintegrating agents is questionable. Similar results for Silvacon, wood flour, and methylcellulose have been reported (2).

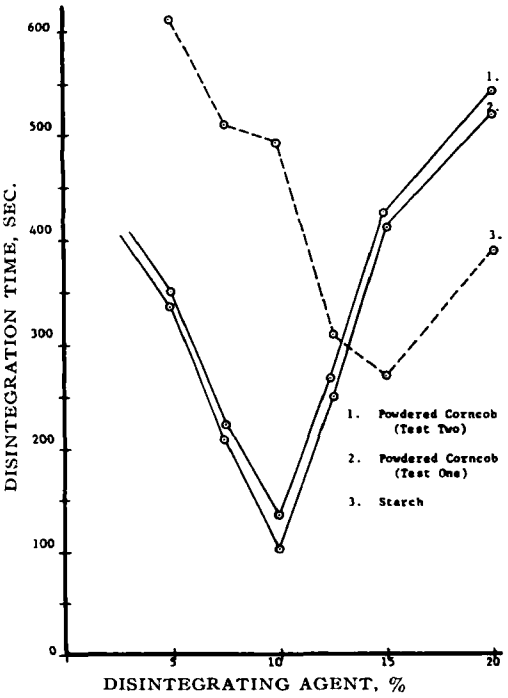

Fig. 2.—Comparison of corncob and starch as disintegrating agents in lactose tablets.

Table I.—Effect of Particle Size of Powdered Corncob on Tablet Disintegration Time

Particle Size, U.S.P. Mesh No.	Time Required to Disintegrate (Av., in sec., for 12 tablets)
No. 60	410
No. 40	95
No. 20	530
No. 14[a]	—

[a] Difficulties encountered in manufacture of tablets.

Normally it would be expected that the 10% concentration would produce a faster disintegration time than the 5% concentration. This was evidenced with corncob, starch, and wood flour. A reversal of this normality was observed with methylcellulose, redwood bark, and the Silvacon products. Billups and Cooper (2) have suggested that these substances possessed adhesive or binding properties which retard disintegration as the concentration is increased. They indicate that this effect may be because of the tendency of these substances to form adhesive gels when hydrated. This suggests that there may be an optimum or maximum quantity of these substances beyond which tablet disintegration will be retarded.

Disintegrating Effects of Powdered Corncob.—The second phase of this study concerned the disintegrating action of powdered corncob. A second lactose granulation was prepared to test the reproducibility of the first test series. A comparison of these results to those of earlier tests is shown in Fig. 2. From these data, no significant difference was indicated between the two tests employing powdered corncob, but significant differences were noted between powdered corncob, lactose, and starch.

Once the reproducibility of corncob had been established, a series of tests was conducted to determine the optimum particle size of powdered corncob. Tablets were made using a lactose granulation with 10% powdered corncob in No. 60, 40, 20, and 14-mesh sieves. All these tablets were readily compressed except the No. 14 mesh. The tablets in this batch were soft because of the large particle size of the powdered corncob and were easily broken before they could be subjected to the disintegration test. Results of these tests are reported in Table I. Since particles of No. 40 mesh produced the fastest

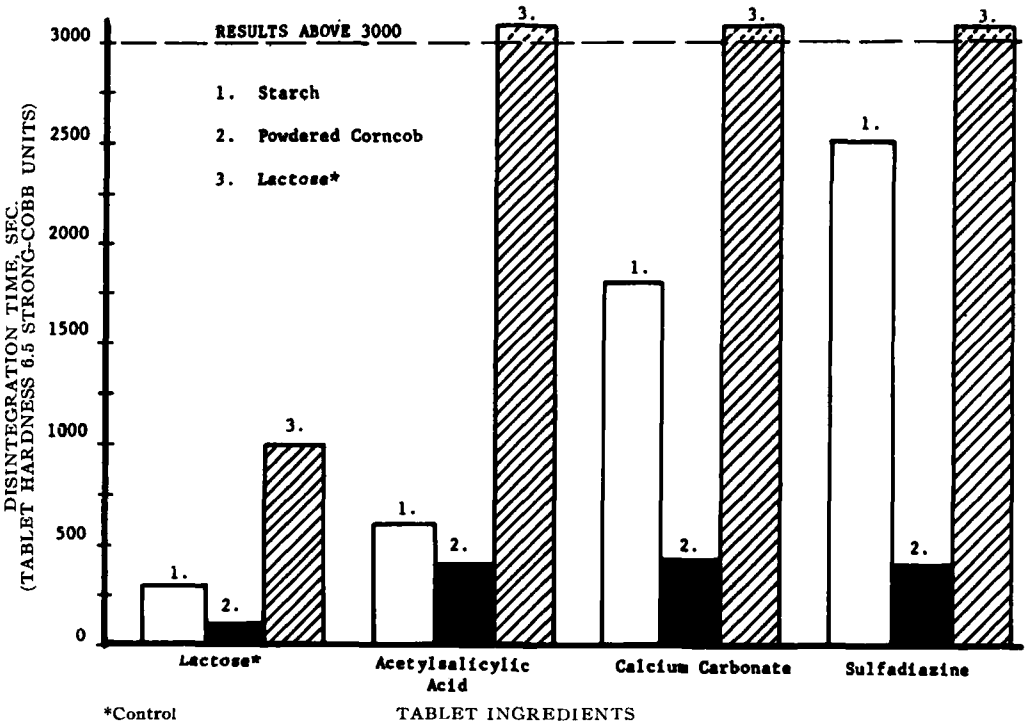

Fig. 3.—Comparison of corncob and starch as disintegrating agents in tablets containing acetylsalicylic acid, calcium carbonate, or sulfadiazine.

tablet disintegration times, this size was employed throughout the remainder of this research.

Thus far, powdered corncob had shown favorable results when used as a disintegrating agent in lactose tablets. Although these results are significant, it remained to be determined if the same results would be obtained in tablets containing active ingredients. Accordingly, powdered corncob was incorporated into granulations of acetylsalicylic acid, calcium carbonate, and sulfadiazine. Lactose was used as the control. A summary of these results is reported in Fig. 3. From these data powdered corncob appeared to be superior to starch in all tablets tested.

Disintegration Effects of Different Parts of Corncob.—The physical appearance of corncob reveals that it is composed of three distinctive parts: the inner area or pith, the middle section or woody substance, and the outer layer or chaff. Burke (15) separated the three layers of corncob and analyzed each layer both quantitatively and qualitatively. His findings, reported in Tables II and III, indicate that starch and cellulose are the two major constituents of corncob. These constituents were further found to vary among each of the three layers. Because of this variability, it was considered necessary to test each of the three parts for tablet disintegrability. The chaff was scraped off with a grater, the pith was bored out with an electric drill, and the remainder of the corncob was ground in a Wiley mill to be used as the wood substance. Each of the separated parts was ground to a fine powder and passed through a No. 40-mesh sieve. The parts were then incorporated into lactose granulations and manufactured into finished tablets. Results shown in Table IV indicate significant differences among the

Table II.—Cellulose Content of Corncob and Its Parts[a]

	As Analyzed, %	Moisture Free, %
Entire corncob[b]	25.15	26.37
Chaff	26.39	28.28
Woody part	25.89	27.30
Pith	33.45	35.85

[a] Burke, G. W., "Some Analytical Data on Corncob," (15), p. 3. [b] The entire corncob in terms of average percentages is composed of chaff 24.97, woody part 73.36, and pith 1.67.

Table III.—Starch Content of Corncob and Its Parts[a]

	Sugar as Dextrose, Gm.	Calcd. to Starch, %
Entire corncob[b]	0.3794	34.15
Chaff	0.3868	34.80
Woody part	0.3828	34.45
Pith	0.3366	30.29

[a] Burke, G. W., "Some Analytical Data on Corncob," (15), p. 3. [b] The entire corncob, in terms of average percentages, is composed of chaff 24.97, woody part 73.36, and pith 1.67.

tablet disintegration times when using different parts of corncob. The three parts of corncob in order of decreasing disintegrating action are pith, woody substance, and chaff. These results raised the question of whether the disintegrating action of powdered corncob or its parts was due solely to the activity of its starch content. It was evident that cellulose must have played an active part since the pith, with the lowest starch content and the highest

TABLE IV.—TABLET DISINTEGRATING ACTIVITY OF CORNCOB AND ITS PARTS

Part(s) Used	Disintegration Time, Av. 12 tablets, sec. Hardness 6.5 Strong-Cobb Units	Hardness 10 Strong-Cobb Units
Entire corncob	95	145
Chaff	291	577
Woody part	56	248
Pith	10	87

TABLE V.—TABLET DISINTEGRATION STUDY OF CELLULOSE *vs.* STARCH

Disintegrant	Disintegration Time, Av. 12 tablets, sec. Hardness 6.5 Strong-Cobb Units	Hardness 10 Strong-Cobb Units
Cellulose	123	245
Cornstarch	300	495
1:1 Parts of cellulose + starch	86	110

cellulose content of all parts, showed the best tablet disintegration time by a large margin.

Role of Cellulose in the Disintegrating Activity of Powdered Corncob.—The third phase of this study was conducted to find the role that cellulose played in the disintegrating activity of powdered corncob. The results from previous tests indicated that cellulose was partially or fully responsible for the disintegrating activity of corncob. To test cellulose as a tablet disintegrating agent, three batches of lactose tablets containing cellulose,[4] starch, and a combination of cellulose and starch were manufactured. Granulations containing 10% starch, 10% cellulose, and a 10% mixture of equal parts of cellulose and cornstarch were incorporated into three tablet formulations. The disintegration times for these three batches are summarized in Table V. These data indicate that cellulose has better disintegrating action than cornstarch. It further shows that a combination of equal parts of cornstarch and cellulose is superior to either of the separate agents. Thus a mutual potentiating effect is shown on the disintegrating action of starch and cellulose. In an earlier study, Crisafi and Becker (16) noticed the same potentiating effect of starch on the disintegrating action of powdered sponge. The potentiating effect of cellulose and starch could be a reasonable explanation for the improved tablet disintegrating activity of powdered corncob over either agent alone.

Mechanism of Disintegrating Action of Powdered Corncob.—The mechanism through which powdered corncob, starch, or cellulose accomplish their disintegrating action is most likely the same. These agents appear to swell when in contact with water and as a result are capable of rupturing tablets in which they are incorporated. Since moisture absorption is an important factor in this action, tests were conducted on powdered corncob, starch, cellulose, a combination of equal parts of starch and cellulose, and lactose (control) to determine the comparative rate of moisture absorption by these agents. One-gram samples of the materials were dried to constant weight at 120 °F. and then placed in a humidity chamber for a 24-hour period. Using a Vapor-Temp[5] humidity chamber the weight gain was calculated by reweighing each sample after the 24-hour interval. A summary of these tests is reported in Fig 4. These results indicate a slight negative correlation between water absorption and tablet disintegration time. Generally, the disintegrating agents which absorbed the most water were those which, when incorporated into tablets, required the shortest times for tablet disintegration.

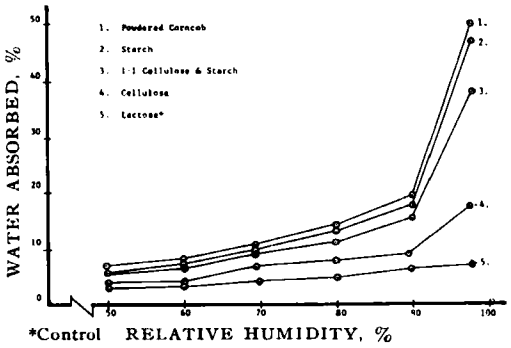

Fig. 4.—Absorptive properties of various disintegrating agents.

It is likely that "swellability" is ultimately the most important single factor in tablet disintegration. In order to swell, however, an agent must first absorb considerable amounts of moisture (17). Figure 4 indicates that powdered corncob, starch, and cellulose each absorbed significant amounts of moisture. Visual examination of these agents indicated considerable swelling compared to the control. Accordingly, each of these agents gave superior tablet disintegration times compared to the control.

CONCLUSIONS

1. Four new substances (powdered corncob, powdered redwood bark, Silvacon 495, and Silvacon 515) have been tested as tablet disintegrating agents and compared to other agents previously reported in the literature. Powdered corncob was shown to be a superior disintegrating agent to starch and the other substances tested. In 5 and 10% concentrations powdered redwood bark was shown to be as good as starch. The Silvacon products were better than the control in 5 and 10% concentrations but not as effective as starch.

2. The disintegrating action of corncob was superior to that of starch in tablets containing soluble, partially soluble, and insoluble material.

3. Results obtained with powdered corncob were reproducible in both placebo and ingredient-containing tablets.

4. The three physically distinct layers of corncob (chaff, woody substance, and pith) were shown to produce significantly different disintegration times. Pith showed superiority over the other two parts.

5. Cellulose was found to be a better tablet disintegrating agent than cornstarch in lactose tablets.

6. Separate and combined effects of starch and cellulose as tablet disintegrating agents were studied.

[4] Stanocel brand of microcrystalline cellulose supplied by the Stanley Drug Co., Portland, Ore., was used in this study.

[5] Marketed by Blue M Electric Co., Blue Island, Ill.

The combined action was shown to be superior to the separate action of either agent alone. This indicates a potentiation effect between these two agents.

7. The mechanism of action through which powdered corncob, starch, and cellulose accomplish their disintegrating action is most likely the same. These agents swell when in contact with water and will rupture the tablets in which they are incorporated. To swell, however, it would appear that an agent must first absorb considerable amounts of moisture. Powdered corncob, starch, and cellulose-starch combinations were shown to absorb significant amounts of moisture. Cellulose absorbed less moisture than starch and corncob but more moisture than the control.

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Notes

Rapid Visual Assay for Penicillinase Concentrates

By FRANCES W. BOWMAN

Enzyme activity is measured by the time required to solubilize procaine penicillin G.

THE LITERATURE abounds with assay methods for enzyme penicillinase, but most are complicated and time consuming. Among these are the microbial assay of Bowman and Holdowsky (1) and various iodometric titrations (2–4). The manometric (5) and optical rotation (6) procedures are accurate but require special equipment. Pollock (7) suggested a rapid approximate assay based on the time required to decolorize a known quantity of iodine in the presence of sufficient penicillin substrate. Ghosh and Borkar (8) recently described an assay incorporating this idea.

A new and simple assay for penicillinase activity has been developed by using a different approach. Enzyme activity is measured by the time required to solubilize completely 300 mg. crystalline procaine penicillin G in pH 7.0 buffer solution. The procaine salt of penicillin G has a solubility in buffer of about 5 mg./ml.; but as quickly as the soluble portion is converted by the enzyme to penicilloic acid, more penicillin goes into solution until finally it is all dissolved.

No requirements of potency have been established for penicillinase concentrates, but for sterility testing of preparations containing penicillin (9) a solution of the enzyme should have at least 4000 Levy units per ml. One Levy unit of penicillinase inactivates 59.3 units (35.6 mcg. or 10^{-7} moles) of penicillin G in 1 hour when the substrate is in sufficient concentration to maintain a zero-order reaction. The test to be described can measure the penicillinase potency of solutions containing approximately 4000–40,000 Levy u./ml., and within this range the solubilization time is a linear function of the dose.

Received September 18, 1962, from the Division of Antibiotics, Food and Drug Administration, U. S. Department of Health, Education, and Welfare, Washington, D. C.
Accepted for publication October 9, 1962.

EXPERIMENTAL

Reagents.—U.S.P. procaine penicillin G, Clark and Lubs 15% buffer, pH 7.0: potassium dihydrogen phosphate, 150 Gm.; 10 *N* sodium hydroxide, 47.9 ml., *q.s.* to 1 L. with distilled water. Distilled water. Penicillinase solutions. Equilibrate all reagents at 25° before use.

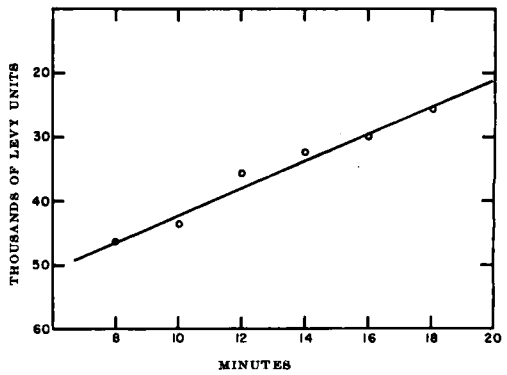

Fig. 1.—Solubilization time for 300 mg. procaine penicillin when tested with various amounts of penicillinase.

The combined action was shown to be superior to the separate action of either agent alone. This indicates a potentiation effect between these two agents.

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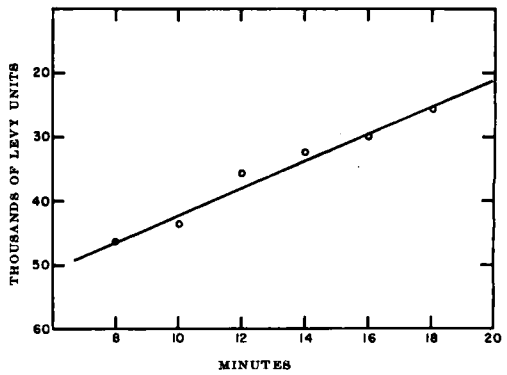

Fig. 1.—Solubilization time for 300 mg. procaine penicillin when tested with various amounts of penicillinase.

TABLE I.—PENICILLINASE ASSAY RESULTS BY TWO METHODS

Levy Units per ml.		Ratio
NaOH Titration Method	Solubilization Method	Col. 2/Col. 1, %
43,296	44,258	102.2
21,075	20,485	97.2
51,676	50,580	98.0
43,415	46,365	106.8
43,836	44,258	101.0
8,430	8,767	104.0
8,430	9,189	109.0
17,703	18,968	107.0
43,346	46,365	106.8
21,075	21,075	100.0
50,580	49,147	97.3
29,505	31,613	107.2
16,860	13,632	81.0
51,170	50,580	98.8
9,273	9,020	97.3
54,205	54,795	101.0
27,145	27,398	101.0
31,613	29,927	94.7
39,452	37,935	96.1
44,848	44,528	99.4
Average		100.1

Assay Procedure.—Suspend 300 mg. of procaine penicillin G in from 9.0–13.0 ml. water in a 20–ml. clear glass bottle with stopper. The volume of water depends on the volume of enzyme sample to be tested. Add 1.0 ml. of the pH 7.0 buffer. Add 1.0–5.0 ml. of the penicillinase test sample to bring the volume to 15.0 ml. and mix. Watch the cloudy suspension and note precisely the time required for it to become completely clear. (A rare small particle or two may be ignored if the solution is substantially clear.) If the time is less than 7 minutes, repeat the test using less enzyme. Samples requiring longer than 20 minutes when 5.0 ml. of enzyme solution is tested contain less than 4000 Levy u./ml. Calculate the Levy units in the sample as follows: Levy units = 63,100–2100 t where t is the solubilization time in minutes. (This relationship is shown graphically in Fig. 1.) Calculate the Levy units per ml. by dividing by the number of ml. of enzyme tested.

Assay of Low Potency Preparations.—Enzymes having very low activity may be assayed by modifying the method. Determine the time required for the enzyme sample to solubilize 300 mg. of procaine penicillin G. A number of hours may be necessary in some cases.

Dilute a previously standardized enzyme concentrate until a dilution is found that requires the same time as the sample to solubilize the same amount of penicillin. Estimate the potency of the unknown from this information. For example, 1.0 ml. of an enzyme solution took 25 hours to solubilize 300 mg. procaine penicillin G. Several dilutions of a stock penicillinase containing 8430 Levy u./ml. were tested by the same method. The dilution requiring 25 hours to solubilize the penicillin contained 422 Levy units per ml. Therefore, it was concluded that the unknown enzyme preparation also contained 422 Levy u./ml.

Application of Assay Method.—A number of penicillinase preparations were tested by the rapid visual method and by a sodium hydroxide titration method (10). The latter is based on the principle that penicilloic acid formed by penicillinase acting on the substrate penicillin may be titrated with sodium hydroxide (11). A solution of soluble sodium or potassium penicillin is adjusted to pH 7.0, the penicillinase (also adjusted to pH 7.0) added, and 0.1 N sodium hydroxide solution added dropwise from a buret, with stirring, to maintain pH 7.0. The reaction is allowed to proceed for 25 minutes and the rate of penicillin destruction calculated from the amount of sodium hydroxide consumed. The rate of inactivation is used to calculate the Levy units.

Results.—As shown in Table I the assays of penicillinase activity by the rapid visual method compare favorably with the results obtained by the sodium hydroxide titration method.

CONCLUSIONS

A simple rapid visual method for determining the enzyme activity of penicillinase concentrates has been presented. The method can be modified to get an approximate assay of low titer preparations. The procedure for enzyme concentrates is especially useful in screening enzyme yields during production runs. It also can be used to estimate the amount of a given enzyme solution to be used in various laboratory tests. When a more precise method with a limited working range is to be used to assign enzyme potency, this method may serve as a preliminary test to establish the approximate activity.

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Acetylation of Sulfamethylthiadiazole

By SHUN-ICHI NAITO† and EINO NELSON

PREVIOUS studies with several sulfonamides, after absorption of a dose and attainment of an apparent equilibrium between drug in blood and drug in other fluids of distribution, show the disposition of the drugs can be described by competing first-order processes (1–4). One of these processes is acetylation of the sulfonamide and the other is excretion of free drug. With those sulfonamides that are excreted in part as N-glucuronides, the rate constant for excretion of free drug includes the rate constant for formation of the N-glucuronide (4–5).

This communication is specifically concerned with the magnitude of the rate constant for acetylation of sulfamethylthiadiazole found in studies conducted in Japan in comparison to the magnitude of this constant found in studies conducted in the U. S. The data from the latter studies has been previously published (2).

EXPERIMENTAL

Normal adult humans ingested 0.5-Gm. tablets of sulfamethylthiadiazole in the morning on overnight fasted stomachs. No food was taken until 2 hours after ingestion of the doses. Preingestion blank urines were obtained and after ingestion of the tablets urine collections were made at appropriate times and assayed for free and total drug by the method of Bratton and Marshall (6). This is the same assay as used in the work done in the U. S. (2). The sulfamethylthiadiazole tablets were 9 mm. in diameter and made with standard concave punches. The tablets were made of finely powdered drug and had the following formula

Sulfamethylthiadiazole	25 parts
Dried potato starch	5 parts
Polyvinyl pyrrolidone 5% in 50% isopropanol	10 parts
Calcium stearate	0.5 per cent

The tablets disintegrated on the average in about 1 minute when tested by the U.S.P. method.

Excretion data were subjected to mathematical analysis by methods previously described (1) to determine the values for the following constants: K, hr.$^{-1}$, the overall rate of removal of drug from the body; k_1, hr.$^{-1}$, the rate constant for acetylation; and k_3, hr.$^{-1}$, the rate constant for excretion of unchanged drug; and f, the fraction of a dose ultimately excreted in the urine as unchanged drug.

TABLE I.—VALUES OF RATE AND OTHER CONSTANTS (TESTS IN JAPAN)

Constant	Subjects H	W	S	T	Mean
K, hr.$^{-1}$	0.589	0.567	0.589	0.658	0.601
k_1, hr.$^{-1}$	0.226	0.225	0.249	0.236	0.234
k_3, hr.$^{-1}$	0.363	0.342	0.340	0.422	0.367
f	0.617	0.604	0.577	0.642	0.610

TABLE II.—MEAN VALUES OF RATE AND OTHER CONSTANTS (TESTS IN THE U. S.)

Constant	Value	Ratio (Japan/U. S.)
K, hr.$^{-1}$	0.514	1.17
k_1, hr.$^{-1}$	0.046	5.11
k_3, hr.$^{-1}$	0.468	0.78
f	0.911	0.670

RESULTS AND DISCUSSION

Table I lists the values of the various constants determined in the experiments.

For the sake of comparison, the mean values of constants found in previously published work with sulfamethylthiadiazole (2) are listed in Table II.

An examination of Table II will show that a more than five-fold difference existed between the values of the rate constant for acetylation obtained in the two sets of experiments. The t test showed a highly significant difference ($P < 0.01$) between the two sets of values of the rate constants. However, there was significantly greater variance in the set obtained in the studies conducted in the U. S. as compared to the variance in the set obtained in Japan.

It is difficult to assign a reason for the difference in k_1 found in the two sets of tests. In the tests conducted in the U. S. (2), two of the test subjects were of Japanese ancestry but their rate constants for acetylation were of the same order as for subjects of Caucasian ancestry. The average age of the test subjects in the U. S. tests was 30 years and in the Japan tests, 20 years. It does not seem likely that the difference could be attributable to this small difference in average age. Both sets of tests were conducted in the spring of the year. Differences in diet seem the most likely explanation, but it is difficult to accept this since first-order kinetics were followed.

Received September 6, 1962, from the Kyoto College of Pharmacy, Kyoto, Japan, and the Pharmacokinetic Laboratory, School of Pharmacy, University of Buffalo, Buffalo 14, N. Y.

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† Present Address: Kyoto College of Pharmacy, Kyoto, Japan.

Note Added in Proof.—It recently came to the attention of one of us (E.N.) that 90% of Nisei Japanese and presumably native Japanese are rapid phenotypes with respect to the rate with which they inactivate isoniazid [Harris, H. W., Knight, R. A., and Selin, M., *J., Amer. Rev. Tubercul.*, **78**, 944(1958)]. Inactivation of this drug is mainly by acetylation. Further, humans may be grouped in rapid and slow categories with respect to the rate with which they acetylate sulfadimidine and rapid acetylators of sulfadimidine are rapid inactivators of isoniazid [unpublished work of D. A. Price Evans, Department of Medicine, University of Liverpool; cited by Clark, C. A., *J. Pharm. Pharmacol.*, **14**, (Suppl.), 20T(1962)]. The possibility exists that the results we report might represent the same phenomenon. Absence of difference in acetylation rate in the earlier U.S. tests may have been due to chance.

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Isolation of β-Sitosterol, β-Sitosteryl-D-Glucoside, and Palmitic Acid from Coastal Bermuda Grass and Orchard Grass

By E. D. WALTER

β-Sitosterol and β-sitosteryl-D-glucoside were isolated from coastal Bermuda grass and from orchard grass, and identified by infrared spectra and physical constants on the sterol and the acetate. Palmitic acid was isolated from the saponifiable fractions. No saponin was detected.

BERMUDA grass and orchard grass were investigated for saponins by means of the procedure used to isolate saponin from ladino clover (1). Material was obtained which gave a Liebermann-Burchard color test suggesting sterols rather than saponin. A study of this material showed that it contained β-sitosterol, β-sitosteryl-D-glucoside, and palmitic acid. Repeated experiments were carried out on 1-Kg. quantities of dehydrated meal to accumulate enough material for identification. Results were practically identical with either of these grasses. No saponin was obtained by this method from these grasses.

EXPERIMENTAL

The dehydrated meal (1 Kg.) was soaked with about 4 L. of warm water for 2 hours. The water was filtered through cloth with suction and discarded. The hydrated meal was covered with 95% alcohol (5–6 L.) and allowed to stand for a day or more. The extract was filtered with suction, and the alcohol extraction was repeated. The combined alcohol extracts were clarified with charcoal (10 Gm./L.), and concentrated to about 1.5 L. The concentrate was extracted with ether in a separator. The ether was evaporated and the residue was saponified with alcoholic potassium hydroxide. The alkaline mixture was extracted with ether, and the ether solution was evaporated to near dryness.

β-Sitosteryl-D-glucoside.—To the unsaponifiable residue from the ether extract, about 100 ml. of skellysolve-B was added. A small quantity of the material was insoluble and was filtered. The residue was washed with acetone, leaving a white product, m.p. 280°. Yield, 90 mg./Kg. dry meal. The material recrystallized from methanol, m.p. 296° (with dec.), showed an infrared spectrum that was in good agreement with that presented by Morris and Lee (2) for β-sitosteryl-D-glucoside.

Hydrolysis of the glucoside with 2% sulfuric acid by the method of Thornton, *et al.* (3), gave β-sitosterol, m.p. 137°, with an infrared spectrum in good agreement with that reported in the literature (2), and glucose which was detected by paper chromatography after the acid had been removed by barium carbonate.

β-Sitosterol.—The skellysolve-B-soluble part of the unsaponifiable material was put on a column of deactivated alumina. The sterol was eluted with 3% acetone in skellysolve-B, and the solvent removed. The sterol crystallized from methanol yielded about 200 mg./Kg. of dry meal, m.p. 137° [lit. 136–137° (4)]. The infrared spectrum was in agreement with that reported (2) for β-sitosterol.

β-Sitosteryl Acetate.—The sterol was heated with acetic anhydride for 30 minutes. The mixture was poured into ice water, and the fluffy product was filtered and washed with water. The dried product was recrystallized from methanol, m.p. 127°; $[\alpha]_D^{25}$ − 38.4° (in chloroform, c = 1.11, *l* = 2), [lit. (4) $[\alpha]_D$ − 38° in chloroform].

Anal. Calcd. for $C_{31}H_{52}O_2$: C, 81.52, H, 11.48. Found: C, 81.6, H, 11.4.

Palmitic Acid.—After ether extraction of the sterols, the aqueous saponifiable portion was acidified with hydrochloric acid and again extracted with ether. The acid was washed out of the ether extract with water and the ether solution was clarified with charcoal. The solution was concentrated to near dryness, then dissolved in methanol. On standing, a white crystalline solid separated. Recrystallization from methanol gave white flakes, m.p. 63°. Titration with 0.1*N* sodium hydroxide gave an equivalent weight of 260 (palmitic acid, 256.32). A gas chromatogram showed that the retention times of this material and that of authentic palmitic acid were identical. Likewise, the infrared spectra of this material and that of authentic palmitic acid were identical.

A minute quantity of material, m.p. about 90°, was obtained before saponification of the ether extract, which suggests that at least part of the sterol occurred as β-sitosteryl palmitate.

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Reference to a company or product does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

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Stabilization of Tetrazolium Blue Assay for Triamcinolone

By PETER ASCIONE and CARL FOGELIN

The substitution of chloroform to the extent of 60 per cent for the pure ethanol normally used in performing the tetrazolium blue assay of triamcinolone produces a stable color in both the sample solution and the reagent blank. Stable color development is achieved within 20 minutes for triamcinolone and its acetonide and diacetate derivatives. The reagent blank reaches a constant value within 10 minutes. In addition, stabilization of the tetrazolium blue reaction overcomes the major objection to its general use and results in a more favorable comparison with triphenyl tetrazolium as the reagent of choice. While the work reported is confined to triamcinolone (9α-fluoro-11β,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20 dione) and two of its derivatives, it appears reasonable to assume that the technique described could be extended to the assay of other ketol steroids.

THE APPLICATION of tetrazolium salts for the determination of ketol steroids was presented by Mader and Buck (1). Smith and Halwer (2) applied this reaction to the determination of triamcinolone alcohol, acetonide, and diacetate. Tetrazolium blue was the specific reagent used in this work. Johnson, King, and Vickers (3) reported that the use of tetrazolium blue as an analytical reagent is complicated by the fact that neither the reagent blank nor the sample achieve a stable color within a reasonable time. The problem was regarded as serious enough by these authors to state a general preference for triphenyl tetrazolium since their observations indicated that this compound did not have this undesirable characteristic. However, tetrazolium blue does show better resistance to attack by oxygen (3) and is approximately twice as sensitive a reagent as triphenyl tetrazolium (1). The work described below allows one to take advantage of the desirable features of tetrazolium blue by eliminating the continuous increase in absorbance of the sample and reagent blank.

DISCUSSION

Origin of Investigation.—The use of chloroform to stabilize the blue-colored formazan produced during the reduction of tetrazolium blue by triamcinolone originated as a result of the application of this solvent to the extraction of triamcinolone acetonide from ointment preparations. The use of chloroform for such extractions is not uncommon (4, 5). However, the chloroform is removed by evaporation prior to assay. While attempting to reduce assay time by eliminating this step, it became obvious that not only could the color reaction be carried out in the presence of chloroform, but also the resulting solutions exhibited better stability than when the chloroform was absent. Subsequent work was carried out to investigate systematically this observation.

Effect of Chloroform on Color Development.—Samples of triamcinolone alcohol, acetonide, and diacetate were dissolved in ethanol. Equal aliquots were transferred to a series of flasks for each of the steroids tested. Varying amounts of chloroform were then added to each sequence and the tetrazolium blue assay completed. Table I gives the data obtained. After examination of these data, it was concluded that a 60% chloroform content represented the concentration of chloroform that would yield the maximum color response consistent with the primary purpose of stabilizing the reaction.

Rate of Color Development in 60% Chloroform.—Assays were performed using the 60% chloroform level to determine the amount of time needed for each of the compounds in question and for the reagent blank to reach a plateau in their absorbance values. An identical set of assays was performed in ethanol alone to provide comparative data between the two solvents. Table II summarizes the results obtained.

It was concluded that the absorbance values obtained between 20 and 30 minutes (after color development was initiated in the chloroform–ethanol mixture) could safely be regarded as having achieved a stable maximum value. This should conservatively estimate the span of time suitable for accurate quantitation. For example, data in Table I indicate at least 1 hour of color stability when triamcinolone alcohol is being tested, and 2 hours' stability for the reagent blank. In addition, 20 minutes is more than sufficient time for maximum color development except for triamcinolone alcohol as shown in Table II.

Linearity of Response.—Standard calibration curves were prepared for all three steroids tested. Typical Beer's law plots were obtained over the concentration range of 1 mcg./ml. to 5 mcg./ml. for all three compounds. The data are presented in Fig. 1.

Effect of Light.—Sensitivity to light remains a problem in the performance of this assay. The use of low actinic glassware as noted by Johnson, *et al.* (3), or storage in the dark until the absorbance values are to be determined, is necessary.

EXPERIMENTAL

Assay Reagents.—(a) Tetramethylammonium hydroxide, supplied as a 10% aqueous solution. Prepare a working solution by diluting 10 ml. to 50 ml. with 95% ethanol; (b) Tetrazolium blue, dissolve 350 mg. in 100 ml. of 95% ethanol; (c) 95% ethanol, essentially aldehyde-free; (d) chloroform, reagent grade.

Assay Procedures.—Prepare a chloroform solution of the steroid to be assayed using a concentration of 5 mcg./ml. for triamcinolone alcohol[1] and diacetate or 10 mcg./ml. for the acetonide. Transfer 15 ml. of the chloroform solution to a 25-ml. low actinic volumetric flask. At the same time prepare a standard of the steroid being assayed to carry through the test. A reagent blank is also prepared by adding 15

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[1] Triamcinolone alcohol is more easily dissolved in ethanol than chloroform. Initial solution should be made in ethanol and a second dilution at 5 mcg./ml. in chloroform prepared

TABLE I.—ABSORBANCE VALUES AT 520 mμ SHOWING THE EFFECT OF THE CHLOROFORM-TO-ETHANOL RATIO ON COLOR DEVELOPMENT[a]

Chloroform, %	Triamcinolone Alcohol, Reaction Time, Min. 20	Triamcinolone Alcohol, Reaction Time, Min. 60	Triamcinolone Acetonide, Reaction Time, Min. 20	Triamcinolone Diacetate, Reaction Time, Min. 20	Reagent Blank, Reaction Time, Min. 20	Reagent Blank, Reaction Time, Min. 60	Reagent Blank, Reaction Time, Min. 120
0	0.328	0.461	0.243	0.224	0.061	0.086	0.102
10	0.408	...	0.310	0.345	0.063	0.081	...
20	0.464	0.513	0.350	0.390	0.070	0.083	0.099
40	0.498	0.513	0.395	0.408	0.081	0.084	0.084
60	0.482	0.483	0.392	0.394	0.084	0.084	0.084
70	0.460	...	0.380	0.361	0.084	0.084	...
80	0.418	0.418	0.361	0.330	0.084	0.084	0.084

[a] The concentrations of the three compounds tested were 0.003, 0.006, and 0.003 mg./ml. for triamcinolone alcohol, acetonide, and diacetate, respectively.

TABLE II.—ABSORBANCE VALUES AT 520 mμ SHOWING THE RATE AND STABILITY OF COLOR DEVELOPMENT IN 60% CHLOROFORM-ETHANOL *vs.* ETHANOL ALONE[a]

Time, min.	Triamcinolone Alcohol, Et.OH	Triamcinolone Alcohol, Et.OH/$CHCl_3$	Triamcinolone Acetonide, Et.OH	Triamcinolone Acetonide, Et.OH/$CHCl_3$	Triamcinolone Diacetate, Et.OH	Triamcinolone Diacetate, Et.OH/$CHCl_3$	Reagent Blank, Et.OH	Reagent Blank, Et.OH/$CHCl_3$
5	0.320	0.730	0.230	0.560	0.280	0.535	...	0.078
10	0.505	0.785	0.390	0.575	0.450	0.562	...	0.085
15	0.605	0.792	0.488	0.575	0.535	0.565	0.091	0.085
20	0.665	0.795	0.540	0.575	0.585	0.565	0.176	0.085
25	0.700	0.795	0.565	0.575	0.610	0.565	0.210	0.085
30	0.730	0.797	0.580	0.575	0.635	0.565	0.268	0.085

[a] The concentrations of the three compounds tested were 0.006, 0.012, and 0.006 mg./ml. for triamcinolone alcohol, acetonide, and diacetate, respectively.

ml. of chloroform to a third 25-ml. volumetric flask. All three flasks are taken through the following procedure.

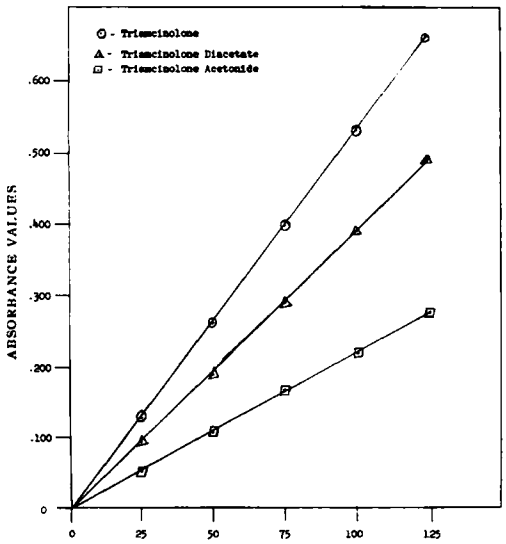

Fig. 1.—Standard calibration curves for triamcinolone, triamcinolone diacetate, and triamcinolone acetonide.

Add 1 ml. of tetrazolium blue solution to each of the flasks, followed immediately by 1 ml. of the tetramethylammonium hydroxide working solution. Bring all flasks to volume with 95% ethanol. Mix well and allow the flasks to stand 20 minutes. Determine the absorbance values for each of the solutions at 520 mμ in any suitable spectrophotometer using ethanol as the reference solution. The value of the steroid content of the unknown samples may then be calculated by comparison with the standard after the reagent blank is subtracted from both.

APPLICATION OF PROCEDURE

This procedure has been applied to routine determination of the steroidal content of a wide variety of pharmaceutical formulations of all three steroids. Tablets, creams, ointments, and suspensions have been successfully assayed using this method. The chloroform often serves a dual purpose by assisting in extracting the steroid from the formulation as well as stabilizing the color reaction.

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Estrogenic Strength of 5-(*p*-Hydroxyphenyl)-cyclohexanedione-1,3

By PHILIPPOS E. PAPADAKIS† and THEODORE J. URBAN

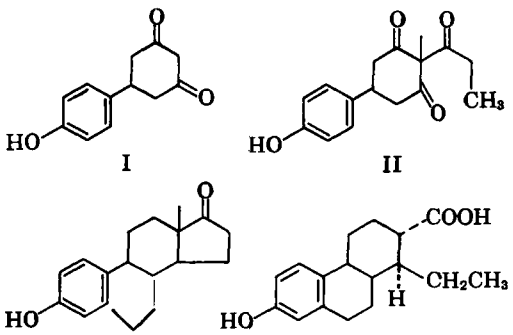
A technique is described for detecting the estrogenic activity of a nonsteroidal compound, 5-(*p*-hydroxyphenyl)-cyclohexanedione-1,3. Under the conditions of this experiment, the uterine weight assay indicated that 0.25 mg./ml. of this compound has estrogenic activity of 5.0 I.U. and 0.75 mg./ml. of the same compound has estrogenic activity of 15 I.U. Vaginal smears indicated that this compound gave a positive response.

BESIDES the natural estrogenic substances, there are synthetic compounds which, although they do not have the steroid ring system, exhibit estrogenic activity. Examples of such synthetic compounds which differ considerably in structure from the natural estrogens are stilbestrol, hexestrol (1), doisynolic acid and its stereoisomers (2–4).

This paper presents the bioassay of an estrogenic compound, 5-(*p*-hydroxyphenyl)-cyclohexanedione-1,3 (I) (5), surnamed by students at Creighton "papadakisone."

Derivatives of I having similarities to compounds of physiological importance have been synthesized. Some of these have been reported (6–8). A C-acyl derivative (7), represented by II, may be considered as an incomplete ring system of estrone (III). Doisynolic acid (IV) is also an incomplete ring system of estrone (III). Since papadakisone (I) is an incomplete ring system of estrone, it seemed reasonable to investigate first its estrogenic activity and then in later experiments proceed to examine the activity of other derivatives which have closer structural resemblance to estrone ex. (II).

The assay of papadakisone is based on an increase of the uterine weight of rats. A typical vaginal smear technique was also used, but since Stob, *et al.* (9), and Rubin, *et al.* (10), indicate that the uterine weight assay is more sensitive than the vaginal smear method, the estimation of estrogenic strength in this experiment is based on the uterine weight assay.



MATERIALS AND METHODS

The test animals used for assay purposes were ovariectomized Holtzman white rats. After the gonads were removed, 14 to 21 days elapsed to allow for uterine atrophy. The rats were killed 24 hours after the last subcutaneous injection of the test material. The rats received 0.1-ml. injections of the aqueous solution of papadakisone every day for 6 days. The solution was made by adding 1 ml. of 6 *N* sodium hydroxide which converts the compound to its soluble salt. The strength of the solution of the dosages used corresponded to 0.25 mg./ml. and 0.75 mg./ml., respectively. The commercial estrogen[1] was used daily in dosages of 10, 25, and 50 I.U. per injection for 6 days (see Table I). The volume of the oil solution of estrogen used for each injection was 0.1 ml. The uteri were weighed using a Roller-Smith precision balance. The uterine weights of the test animals treated with various dosages of commercial estrogen and the increase in uterine weight was used to indicate estrogenic activity.

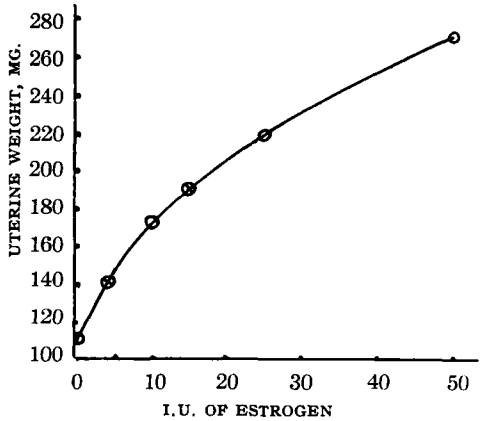


Fig. 1.—Uterine weight response to various dosages of estrogenic substances. ○ = Urestrin; ⊕ = papadakisone.

RESULTS

Data on the changes in uterine weight due to different dosages of estrogen and papadakisone are presented in Tables I and II. Data showing vaginal smear responses are presented in Table III.

In order to establish a means of reference for papadakisone-induced increases in uterine weight, a standard response curve (Table I) was determined using subcutaneous injections of estrogen. The rats receiving papadakisone all exhibited uterine weights that were heavier than the weights of the controls, and these differences also applied when the uterine weights were expressed as a percentage of body weight.

The work with vaginal smears indicates that papadakisone gives a positive response (Table III).

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[1] Marketed as Urestin by The Upjohn Co.

TABLE I.—STANDARD DOSE RESPONSE TO SUBCUTANEOUS INJECTIONS OF ESTROGEN[a] BASED ON UTERINE WEIGHT RESPONSE

Estrogenic Hormone (I.U.)	No. of Animals	Mean Body Wt. (Gm.)	Range of Rats' Wt. (Gm.)	Body Wt., %	Mean Uterine Wt. (mg.)
None	15	228.40	233.4–261.0	0.0503	115.10
10 I.U.	9	247.40	233.2–266.8	0.0702	173.62
25 I.U.	8	248.76	225.4–272.9	0.0893	220.52
50 I.U.	10	245.06	223.4–263.9	0.1101	269.88

[a] Urestin.

TABLE II.—RESPONSE TO SUBCUTANEOUS INJECTIONS OF PAPADAKISONE BASED ON UTERINE WEIGHT RESPONSE

Hormone, mg./ml.	No. of Animals	Mean Body Wt. (Gm.)	Range of Rats' Wt. (Gm.)	Body Wt., %	Mean Uterine Wt. (mg.)
None	10	218.87	165.1–277.9	0.0431	94.46
0.25	10	239.00	217.8–255.3	0.0589	140.96
0.75	10	244.40	230.4–260.4	0.0781	190.88

TABLE III.—RESPONSE TO SUBCUTANEOUS INJECTIONS OF ESTROGENIC COMPOUNDS BASED ON THE VAGINAL SMEAR RESPONSE

Treatment	No. of Animals	No. of Positive Smears	Positive, %
None	10	0	0
0.25 mg./ml.[a]	10	10	100.0
0.75 mg./ml.[a]	10	8	80.0
50 I.U.[b]	12	11	91.88

[a] Papadakisone. [b] Urestrin.

DISCUSSION

It seems quite safe to say that the nonsteroidal compound (called papadakisone here) has estrogenic activity. This is evidenced by positive vaginal smears and an increase in uterine weights. Comparing the responses of the experimental substance to the responses of I.U. of estrogen, the results indicate that 0.25 mg./ml. of papadakisone has an estrogenic activity in the neighborhood of 5.0 I.U. (Fig. 1). The dose of 0.75 mg./ml. of papadakisone causes a uterine weight response about that of a 15 I.U. dosage of estrogen. In searching the literature it was found that Lespagnol and Schmitt (11) compared the estrogenic activity of 5-(*p*-methoxyphenyl)-cyclohexanedione-1,3 to that of estrone and found the ratio of 1/500.

When treated with sodium hydroxide solution structure I is converted to its sodium salt, so that the estrogenic activity measured should be considered as due to the sodium salt of I.

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Isolation of Lupeol from *Sweetia panamensis*

By THOMAS J. FITZGERALD, JACK L. BEAL, and JULES B. LAPIDUS

SWEETIA PANAMENSIS Benth. is one of several medicinal Central American plants currently being investigated in this laboratory. For the purpose of facilitating subsequent extractions, the bark of this plant was defatted with skellysolve B, and from this extract the triterpene, lupeol, was isolated. Thus *S. panamensis* may be added to the already extensive list of plants in which this compound is found.

The isolation of lupeol from this particular source is of some historical interest. Thompson (1) reported the isolation from *S. panamensis* of a compound which according to his description is quite similar to lupeol. Indeed, when we repeated his work, lupeol was isolated from an ethanolic extract of the bark. Inasmuch as Thompson's investigation was reported in 1884 this would seem to constitute the first known isolation of lupeol rather than that of Schulze and Steiger in 1889 (2).

Also, this investigation has shown that the lupeol occurs in the bark of this plant as the free alcohol. The triterpene was characterized through formation of the acetate, benzoate, and by use of infrared spectra.

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Accepted for publication November 7, 1962.
This investigation was supported by a research grant from the A. H. Robins Co., Richmond, Va.

EXPERIMENTAL

Isolation of Lupeol.—The bark[1] was powdered

[1] Obtained from S. B. Penick and Co.

TABLE I.—STANDARD DOSE RESPONSE TO SUBCUTANEOUS INJECTIONS OF ESTROGEN[a] BASED ON UTERINE WEIGHT RESPONSE

Estrogenic Hormone (I.U.)	No. of Animals	Mean Body Wt. (Gm.)	Range of Rats' Wt. (Gm.)	Body Wt., %	Mean Uterine Wt. (mg.)
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10 I.U.	9	247.40	233.2–266.8	0.0702	173.62
25 I.U.	8	248.76	225.4–272.9	0.0893	220.52
50 I.U.	10	245.06	223.4–263.9	0.1101	269.88

[a] Urestin.

TABLE II.—RESPONSE TO SUBCUTANEOUS INJECTIONS OF PAPADAKISONE BASED ON UTERINE WEIGHT RESPONSE

Hormone, mg./ml.	No. of Animals	Mean Body Wt. (Gm.)	Range of Rats' Wt. (Gm.)	Body Wt., %	Mean Uterine Wt. (mg.)
None	10	218.87	165.1–277.9	0.0431	94.46
0.25	10	239.00	217.8–255.3	0.0589	140.96
0.75	10	244.40	230.4–260.4	0.0781	190.88

TABLE III.—RESPONSE TO SUBCUTANEOUS INJECTIONS OF ESTROGENIC COMPOUNDS BASED ON THE VAGINAL SMEAR RESPONSE

Treatment	No. of Animals	No. of Positive Smears	Positive, %
None	10	0	0
0.25 mg./ml.[a]	10	10	100.0
0.75 mg./ml.[a]	10	8	80.0
50 I.U.[b]	12	11	91.88

[a] Papadakisone. [b] Urestrin.

DISCUSSION

It seems quite safe to say that the nonsteroidal compound (called papadakisone here) has estrogenic activity. This is evidenced by positive vaginal smears and an increase in uterine weights. Comparing the responses of the experimental substance to the responses of I.U. of estrogen, the results indicate that 0.25 mg./ml. of papadakisone has an estrogenic activity in the neighborhood of 5.0 I.U. (Fig. 1). The dose of 0.75 mg./ml. of papadakisone causes a uterine weight response about that of a 15 I.U. dosage of estrogen. In searching the literature it was found that Lespagnol and Schmitt (11) compared the estrogenic activity of 5-(*p*-methoxyphenyl)-cyclohexanedione-1,3 to that of estrone and found the ratio of 1/500.

When treated with sodium hydroxide solution structure I is converted to its sodium salt, so that the estrogenic activity measured should be considered as due to the sodium salt of I.

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Isolation of Lupeol from *Sweetia panamensis*

By THOMAS J. FITZGERALD, JACK L. BEAL, and JULES B. LAPIDUS

SWEETIA PANAMENSIS Benth. is one of several medicinal Central American plants currently being investigated in this laboratory. For the purpose of facilitating subsequent extractions, the bark of this plant was defatted with skellysolve B, and from this extract the triterpene, lupeol, was isolated. Thus *S. panamensis* may be added to the already extensive list of plants in which this compound is found.

The isolation of lupeol from this particular source is of some historical interest. Thompson (1) reported the isolation from *S. panamensis* of a compound which according to his description is quite similar to lupeol. Indeed, when we repeated his work, lupeol was isolated from an ethanolic extract of the bark. Inasmuch as Thompson's investigation was reported in 1884 this would seem to constitute the first known isolation of lupeol rather than that of Schulze and Steiger in 1889 (2).

Also, this investigation has shown that the lupeol occurs in the bark of this plant as the free alcohol. The triterpene was characterized through formation of the acetate, benzoate, and by use of infrared spectra.

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This investigation was supported by a research grant from the A. H. Robins Co., Richmond, Va.

EXPERIMENTAL

Isolation of Lupeol.—The bark[1] was powdered

[1] Obtained from S. B. Penick and Co.

in a Wiley mill, and 1000 Gm. was extracted to exhaustion in a Soxhlet apparatus with 2.5 L. of skellysolve B. The light green solution was reduced to dryness *in vacuo* and yielded a light greenish-yellow residue weighing 20.1 Gm. The residue, when subjected to the Liebermann-Burchard test, gave the characteristic red color of lupeol.

Ten grams of this residue was refluxed for 3 hours with 250 ml. of alcohol containing 12 Gm. of sodium hydroxide. After cooling, the brown solution was poured into 300 ml. of water, and a flocculent yellow precipitate formed. The alcohol was evaporated on a steam bath, and after filtering the suspension, the precipitate was washed with 500 ml. of water. After drying at 105° for 12 hours the crude unsaponifiable material weighed 5.7 Gm. Recrystallization from ethanol gave needles, m.p. 212–213°.[2] When this material was mixed with an authentic sample of lupeol[3] (m.p. 214.5°) and melted, no depression occurred. Further purification was achieved by preparing the acetate and saponifying. This procedure gave a product, after recrystallization from ethanol, m.p. 214 to 214.5°; lit. value, 215° (4). The infrared spectrum of this material was identical with that of an authentic sample.

[2] Melting points are uncorrected.

[3] The authentic samples of lupeol and its derivatives were provided by Dr. Jack L. Beal from a previous investigation (3).

Lupeol Acetate.—The acetate was prepared according to Wagner (5). Recrystallization from ethanol gave the compound as needles with a melting point range of 217 to 217.5°; lit. value, 218° (4).

Lupeol Benzoate.—The benzoate was also prepared according Wagner (5). This material was obtained as small platelets when recrystallized from benzene-ethanol (1:5) and gave a melting point range of 264–266°; lit. value, 273–274° (4). The infrared spectrum of this compound was identical with that of an authentic specimen of lupeol benzoate.

Presence of Free Lupeol.—A portion of the skellysolve residue was washed with methanol and then dissolved in chloroform. The needle-shaped crystals which formed on evaporation of the solvent at room temperature, m.p. 211–213°, gave an infrared spectrum which was identical with that of the material obtained by saponification.

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Communications

Absence of 6-Methoxybenzoxazolinone in Uninjured Maize Tissue

Sir:

The peculiar phenomenon has been demonstrated (1) that many living plants contain stable precursors which after the crushing of plants are enzymically decomposed to substances which (or after further chemical or enzymic decomposition) have different biological activities. One of the typical cases is the formation of benzoxazolinone (BOA) and its 6-methoxy derivative (MBOA) in crushed rye and maize plants, respectively. When these compounds were isolated and characterized chemically in this laboratory, it was at first believed that they were present in the intact plants (2). Later on we could relate their formation to the precursors present in these plants. The reaction series is:

precursor, glucoside of 2,4-dihydroxy-7-methoxy- —enzymic→ aglucone —chemical→ 1, 4-benzoxazin-3-one —chemical→ 6-methoxybenzoxazolinone (3, 4).

The last step in the reaction is of an unusual type—a six-membered ring is transformed into a five-membered one by the splitting of formic acid. When hydrolysis of the glucoside is completely prevented in the intact plant, we have never detected any formation of BOA or MBOA.

Smissman, *et al.* (5), recently reported that they found MBOA as an original substance in maize tissue. Quantitative data were not given. Their procedure was complicated, however, and involved some steps during which the formation of MBOA from the aglucone can take place. If the aglucone was already present in the ground plants when they were placed in 95% ethanol, the formation of some MBOA would be expected. Kinetic studies in this laboratory have shown that aglucone of the rye glucoside is converted into BOA at a much higher rate in 80% ethanol than in aqueous solution (6). The rapid conversion of the aglucone of the maize glucoside into MBOA in 95% ethanol solution at 40° is shown in Fig. 1.

The other step at which MBOA could surely be formed from the maize aglucone is the fractiona-

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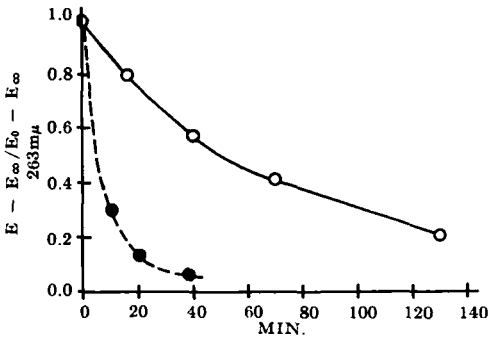

Fig. 1.—Decomposition of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one to 6-methoxybenzoxazolinone in 95% ethanol (solid line) at 40°C. and in 20% ethanol in ether in the presence of Woelm's acid aluminium oxide at 20°C. (broken line).

tion of the ethanol solution after ether extraction on an aluminium oxide (Merck) column. We have observed very rapid decomposition of the aglucone in the presence of this oxide (Woelm's preparation). The result is seen in Fig. 1. If the aglucone was present in the ether solution, MBOA was formed in the experiment of Smissman, *et al.* (5).

A simple and reliable method of inactivating the glucosidase and completely preventing the enzymic hydrolysis of the glucoside is to place the intact plant in ethanol solution (final ethanol concentration 70 to 80%) either at 0° or at a lower temperature for 24 hours. The temperature of the mixture is then allowed to rise to room temperature, after which the mixture is ground in a Waring Blendor and the alcohol evaporated in a vacuum. We made some new estimations of MBOA by immersing intact maize plants (Early Albert and a sugar maize variety) in a carbon dioxide–ethanol mixture (−76°) for 24 hours. The dry residue was extracted with ether, and the ether solution evaporated at about 0°. The residue was dissolved in a small volume of acetone and the solution mixed with a threefold volume of 2% acetic acid in water. The mixture was poured onto a small cellulose powder column and eluted with 2% acetic acid until 70 ml. of eluate was collected. The eluate was freeze-dried, and the residue dissolved in acetone and chromatographed on Whatman No. 1 paper with 2% aqueous solution of acetic acid as solvent. The aglucone has the R_f value 0.74 and MBOA 0.49 (ascending). The spots were detected by U. V. absorption, the aglucone spot also by the formation of the blue-colored complex on spraying with alcoholic ferric chloride solution. Using these methods, we found neither the aglucone nor MBOA in young maize plants (about 2 weeks old). In older plants (1 to 2 months old) grown in the laboratory greenhouse under artificial light, when the tips of many of the leaves were already yellow, small amounts of aglucone (20 mcg./Gm. fresh wt.) were detected (about the amount of aglucone after enzymic hydrolysis see below), but no MBOA. It is possible, but not proved, that the aglucone was formed in the withered parts of the leaves, or that it is generally present in older plants.

When the enzymic hydrolysis of the glucoside was needed for the estimation of the aglucone and glucoside, the plants were ground in a mortar at about 10°. After standing for 1 hour the mixture was evaporated at low temperature and the dry residue extracted with ether and subsequently treated as described above. Five-hundred micrograms of aglucone was found per Gm. fresh wt. in 2-week-old maize plants (Early Albert), and 100 mcg. of aglucone per Gm. fresh wt. in 2-month-old plants. In 1-month-old sugar maize, the amount of aglucone was 200 mcg. per Gm. fresh wt. No MBOA could be detected in these plants either.

On the basis of our previous and present findings, we still consider that uninjured maize plants do not contain MBOA. The presence of free aglucone in small amounts in older maize plants is possible but requires additional evidence.

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Methods of Biochemical Analysis. Vol. XI. Edited by DAVID GLICK. Interscience Publishers, 440 Park Ave. South, New York 16, N. Y. ix +442 pp. 15.5 × 23.5 cm. Price $14.50.

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Progress in Drug Research. Vol. IV. Edited by ERNST JUCKER. Interscience Publishers, 440 Park Ave. South, New York 16, N. Y. 606 pp. 17 × 24.5 cm. Price $30.

Subjects presented in this fourth volume of this survey series discussing the chemical, pharmacological, and clinical aspects of pharmaceutical research include recent developments in therapeutically useful organic sulfur compounds, drug latentiation, antihypertensive agents, structure-activity relationships of local anesthetics, and molecular geometry and mechanism of action of chemical carcinogens. The subjects are presented in the language of the contributor, either English or German. The subject index, also in the language of the respective contributions, includes trade names as well as chemical and trivial names—an excellent feature of the work.

A Survey of Drugs. By HAKIM ABDUL WAHID and H. H. SIDDIQUI. Institute of the History of Medicine and Medical Research, Hamdard Buildings, Delhi 6, India. xv + 168 pp. 16 × 24 cm. Price $1.

A systematic classification of information on indigenous drugs of India and the surrounding regions is presented to bring together available information of the drugs used in Indian systems of medicine which may be of interest to pharmaceutical investigators. The classification is effected through different lists, 19 in all. The lists classifying the indigenous drugs added by Unani physicians to their materia medica, vegetable drugs used exclusively in Unani medicine and the comparable information for Ayurvedic medicine, and drugs according to their uses and action in Unani medicine may be of particular interest. A brief textual section covers the background of the different systems of medicine of the area and helps the reader orient himself when considering the various classifications established.

First International Pharmacological Meeting. Vol. VI. General Editor: BORJE UVNASS. The Macmillan Company, 60 Fifth Ave., New York 11, N. Y. xiii + 330 pp. 16 × 24 cm. Price $15.

A symposium covering biochemistry and pharmacology is reported. The list of 41 author-participants from this and other countries is composed of eminently qualified scientists. The first part is devoted to enzymes that metabolize drugs—excluding, however, antitumor drugs. The second part of the program concerns factors that influence the amount of drug available to the enzymes and factors which change their activity. Specific topics presented include: detoxication mechanisms *in vivo*, demethylation and methylation of drugs and physiologically active compounds, desulfuration, urinary excretion of drugs, duration of action of drugs as affected by tissue distribution, and species differences and individual variations in drug metabolism.

First International Pharmacological Meeting. Vol. VIII. Edited by W. D. M. PATON. The Macmillan Company, 60 Fifth Ave. New York 11, N. Y. xvi + 330 pp. 15.5 × 23 cm. Price $15.

A symposium designed to concentrate on the mechanism of action and analytical aspects of drugs active on the central nervous system and various approaches to central nervous system pharmacology is reported. Thirty-one papers fitting within the format described above were selected for publication and these include: inhibitory factors in the brain; central amines and behavorial states: a critique and new data; unusual central depressant properties of a new piperidine compound; drug-induced changes in behavior; and controlled environment: a method of measuring the drug response–behavior in humans.

Encyclopedia of Chemical Technology. Vol. 1. Edited by ANTHONY STANDEN. John Wiley & Sons, Inc., 440 Park Ave. South, New York 16, N. Y. xvi + 990 pp. 19 × 27 cm. Price $45.

The first volume of the second edition promises to be as useful a resource and reference work as the first edition. The aim of this thorough revision, volume by volume, is to provide a comprehensive summary of knowledge on the industrial methods, materials, processes, and equipment of chemical technology. The second edition will be broadened in scope to include information from abroad as well as the United States. The encyclopedia will contain articles on pharmaceuticals and cosmetics. About one-half of the articles deal with single or compound chemical substance.

Infrared Absorption Spectroscopy, Practical. By KOJI NAKANISHI. Holden-Day, Inc., 728 Montgomery St., San Francisco 11, Calif. ix + 233 pp. 18 × 26 cm. Price $8.

Actual infrared curves are presented to acquaint the reader with the range of absorption of the various characteristic absorption bands. Factors which influence band positions and intensities are discussed. The work is a translation of a Japanese book first published in 1960.

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Infrared Absorption Spectroscopy, Practical. By KOJI NAKANISHI. Holden-Day, Inc., 728 Montgomery St., San Francisco 11, Calif. ix + 233 pp. 18 × 26 cm. Price $8.

Actual infrared curves are presented to acquaint the reader with the range of absorption of the various characteristic absorption bands. Factors which influence band positions and intensities are discussed. The work is a translation of a Japanese book first published in 1960.

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August 1963

volume 52 number 8

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The Editor comments

BUT WHERE WAS PHARMACY?

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The activities and responsibilities of many medical and medically-oriented groups were formally presented during the Conference program by an array of distinguished physicians who are active in clinical research, education, government, administration, and industry. In addition, enlightening papers were delivered by nonmedical authorities from government and law.

This writer had the pleasure of attending the Conference as the invited representative from the A.PH.A., and was accompanied by two representatives from the A.PH.A. Scientific Section. As we listened to the program presentations, however, the writer could not help but reflect upon the fact that pharmaceutical scientists were completely absent from the program roster. In view of the fact that the charter of the Commission states that it will exist to "recommend means for expansion of scientific knowledge of the predictability of action of drugs in man," it must be concluded that the Commission does not recognize that the manner in which a specific pharmaceutical dosage form is constructed plays a fundamental role in whether or not the particular drug will be safe and effective when it is administered.

When one considers that the Commission was fathered by an industry which places considerable significance on the variations in safety and effectiveness which may be encountered in finished dosage forms of the same drug substance when manufactured by different firms, then it becomes all the more difficult to understand why this important aspect of drug safety was ignored on an otherwise very comprehensive and enlightening Conference program.

Edward G. Feldmann

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Review Article

Pharmaceutical Sciences—1962. Part II

A Literature Review

By WAYNE McKEEHAN

CONTENTS

[Contents for Part I of this review appeared in the July issue, THIS JOURNAL, 52, 613(1963).]

RADIOISOTOPES

BOUSQUET has reviewed radioisotope applications to cosmetic analysis and product evaluation (536). Methods and apparatus were described for the investigation of aqueous properties and structure of disperse bodies with the aid of radioactive isotopes (537). Appino, *et al.*, presented a radioisotopic method for the evaluation of emulsions which was applicable to either water-in-oil or oil-in-water systems (538). Radioactive tracer techniques were used by Banker to study uniformity of distribution of phosphorus compounds in tablet matrices (539). Scintillation counting was employed for studying adsorption and hydrogenation of radioactive crotonic and vinylacetic acids on thiophene-poisoned palladium (540). Continuous measurement of diffusion coefficients of gases in liquids using glass scintillators made it possible to monitor slow diffusion processes for several hundred hours without disturbing the system by sampling (541). Labeled atoms were also used in the determination of diffusion coefficients of drugs in polymers (542). Radioactive isotopes were used in studying vitamin B_{12} absorption (543), and Ruggiero

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and Skauen have described a chick embryo technique for evaluation of radioactive sodium iodide absorption from various ointment bases (544, 545).

A few papers dealt with the use of radioactive isotopes in pharmaceutical sterilization methods. Sterilization by means of ionizing radiation was reported to be advantageous only for preparations containing compounds such as proteins, enzymes, and steroids which cannot be sterilized by conventional techniques (546). Radiation sterilization of antibiotics (547) and sterilization of ampuled drugs and drug solutions (548) have also been investigated. Schwenker and Vogt discussed the effects of sterilization doses of ionizing radiation on liquid and solid petrolatum (549).

Rames and Bailey studied the chemical effect of high level gamma irradiation on blood glucose *in vitro* (550). A review of the preparation and production control of iodine-131 and colloidal gold-198 was published (551). Two methods for the preparation of tritium-labeled oxytocin were reported (552), and Peng described a method for the preparation of soluble radiophosphate (553). The effects of ionizing radiation on two gelatin fractions were studied (554). When vitamin B_1 hydrochloride crystals were irradiated with Co^{60} γ-rays, a more dense, less hygroscopic material was obtained (555). Radiochemical purity of tritium exchange-labeled barbital was the subject of another paper (556). A review article discussed the application of radioisotopic indicators to paper chromatography (557). Stability of labeled sodium polymetaphosphate was investigated by means of a chromatographic method (558). In studying the mechanism of action of phenolic disinfectants, Judis investigated the release of radioactivity from C^{14}-labeled *Escherichia coli* (559).

Christian, *et al.*, described a 2π liquid scintillation counter for determining the radioactivity of large samples, including man and animals (560). The assembly and operating characteristics of a 4π liquid scintillation detector were reported by Dunavant (561). A Co^{60} γ-irradiator with an unusually high radiation intensity per curie of cobalt has been developed for use in chemical research (562). Rhodes disclosed a simple formula for mass absorption coefficients near the K absorption edge (563), and Evans proposed a simplified self-absorption correction for isotopes emitting weak β-particles (564). Parameters affecting the resolution of a proportional counter have been discussed (565). In another publication, Meade and Stiglitz considered improved solvent systems for liquid scintillation counting of body fluids and tissues (566).

BIOPHARMACEUTICS

Biopharmaceutics is that area of pharmaceutical science primarily concerned with effects of pharmaceutical formulation on the biological activity of medicinal agents. Research in this area requires a certain amount of knowledge in allied fields such as biochemistry, pharmacology, and physiology, to name just a few. Several references in these related areas have been included as part of the review.

Friend discussed the importance of pharmaceutical formulation on the clinical efficacy of drugs and suggested some factors to be considered in the development of pharmaceuticals (567). Problems encountered in the design and presentation of medicinal substances were reviewed (568). Three papers discussed evaluation of new drugs (569), clinical trials of drugs (570), and drug screening and evaluation procedures (571). Pfeiffer described some problems encountered in exploratory trials of new drugs in man and ways of overcoming them (572). The use and abuse of mixtures of active drugs were reviewed (573), while another paper discussed unexpected hazards associated with drugs and chemicals (574).

Automation in biopharmaceutics and related areas was treated by several writers. Applications of data-processing equipment in clinical drug evaluation were reviewed (575). Machine retrieval of pharmacodynamic data was reported by Dietrich (576). An automatic digital computer was employed for the solution of some chemotherapeutic problems (577). Also published was a paper on the use of program-controlled automatic calculators for dosage computations (578).

Bousquet, in a review on the pharmacology and biochemistry of drug metabolism, discussed some of the biochemical reactions undergone by drugs (579). Another writer considered the relation of pharmacology to medicine and pharmacy (580), and contributions to medicine by research in pharmacology were summarized by Riker (581). In a symposium on clinical drug evaluation, Brodie described some of the difficulties in extrapolating data on metabolism of drugs from animal to man; he pointed out that the greatest difficulty is species difference in biotransformation of drugs (582). Several references were cited in a review of methods for studying the anti-inflammatory steroids (583). Kirsner, *et al.*, discussed some problems in the evaluation

of gastrointestinal drugs and ways of overcoming them (584). The effect of intestinal motility on the absorption of sodium in man was investigated (585). Regulation of gastric emptying after meals containing citric acid and salts of citric acid was studied by Hunt and Knox (586).

A theoretical and experimental approach to various facets of drug-receptor interaction has been published (587). In their studies on drug receptors in smooth muscle, Takagi and Iwaki (588) and Iwaki (589, 590) investigated the action of reversible and irreversible antagonists on vascular smooth muscles. A gel filtration method was described for the study of drug-protein binding; quantitative separation of bound and unbound drug was obtained from Sephadex columns (591). Barlow, *et al.*, also measured drug-plasma binding with the aid of Sephadex (592). Semipermeable membranes and electrophoresis were used to study the interaction of sulfapyroxyline and sulfamerazine with serum protein (593). The binding of nitrofurans by proteins was also investigated (594). Scholtan examined the binding of long-acting sulfonamides on human serum proteins with a new small-capacity dialyzator (595), while Scholtan and Schmid showed that the binding of penicillin to serum and tissue proteins was directly related to penicillin concentration but inversely related to temperature (596). Dialysis experiments and radioactive histamine were used in studying histamine binding by heparin (597). Another report described the interactions of xanthine molecules with bovine serum albumin (598).

Effects of Formulation.—Two methods have been described for determining whether patients are actually taking their medication: Swintosky used riboflavin as a marker which could be detected in urine (599), and Ryan, *et al.*, proposed a similar procedure using phenolsulfonphthalein (600). If phenolsulfonphthalein is present, the urine will turn violet when made basic. Aerosol administration of ergotamine tartrate for the treatment of migraine headache was investigated in a study which compared aerosol administration with parenteral injection, oral and sublingual dosage, and rectal suppositories (601). Atropine micro-aerosols and their effects on airway resistance in man were studied (602). In another study a new noscapine formulation for relief of symptoms associated with respiratory tract disorders was also described (603). Schnell and Husa studied the permeability of red corpuscles to water-soluble organic iodine compounds (604), while Shaw and Husa observed the hemolysis of red corpuscles by various substances in the presence of sodium chloride (605). Experimental work on the cause of pain following injection of parenteral pharmaceuticals was published (606, 607). Thoma, *et al.*, examined the effect of bentonite and Veegum on the activity of a series of antiseptics (608). Two papers were published on the subject of physiological availability: one discussed the influence of granulating agents on biological availability of tablet medication (609); the other dealt with the relationship between disintegration time and physiological availability of medication in long-acting tablet formulations (610). Antibacterial activity of mixtures of quaternary ammonium compounds and hexachlorophene was investigated (611).

An *in vitro* method which complies with biological behavior was described for the evaluation of antacids (612), and Newey reported that peptone should be included in artificial gastric juices for antacid testing (613). Clinical and experimental observations on a new antacid were also published (614). Beekman discussed the preparation and properties of some new antacids containing aluminum and magnesium (615, 616). In a study of the influence of common antacids on intragastric pH, dihydroxy aluminum sodium carbonate was found to have a more significant prolonged effect than other compounds investigated (617). Continuous recording of pH by a glass electrode *in situ* was used in assessing the results of diet, antacids, and anticholinergic agents on gastric and duodenal acidity (618). Davison, *et al.*, studied the effects of buffered and unbuffered acetylsalicylic acid on the gastric acidity of normal humans; compared with a control, neither aspirin nor buffered aspirin produced appreciable changes (619). Toxicity of aspirin preparations to the gastrointestinal tract was the topic of another paper (620).

Effects of Physicochemical Properties.—Nelson has discussed physicochemical and pharmaceutic properties of drugs that influence the results of clinical trials (621). The role of ionization constants in the absorption of drugs from the gastrointestinal tract was reviewed (622). An absorption-rate hypothesis, based on physicochemical properties, for the absorption of drugs after subcutaneous implantation was presented by Ballard and Nelson (623). Aqueous/lipid partition coefficient was found to be an important factor in percutaneous absorption of weak electrolytes (624). Treadwell, *et al.*, investigated conditions controlling sterol absorption (625). Hofmann and Borgström suggested that lipids are probably absorbed from the in-

testinal tract as micelles with bile salts acting as transport carriers (626).

Physicochemical factors influencing the absorption of erythromycin and its esters were investigated by Nelson; dissolution rates of erythromycin and some of its esters in fluids simulating gastric and intestinal juices were presented (627). A biological study of some new derivatives of demethylchlortetracycline has been published (628). A report on factors influencing the absorption of griseofulvin from the gastrointestinal tract has also been published (629). Physiological availability of riboflavin and thiamine from "chewable" vitamin products was determined by Morrison and Campbell (630). Of the nine products they studied, six showed incomplete riboflavin availability as measured by urinary excretion. A chromatographic method for testing the availability of ionic iron from iron chelates was published (631). In another article, the use of an iron-sorbitol-citric acid complex in iron-deficiency anemia was disclosed (632). Dettbarn suggested that the cationic form of local anesthetics is intrinsically the most potent (633). A series of papers was published on the relations between physicochemical properties and the action of local anesthetics (634–637). Truitt and Morgan compared gastrointestinal absorption rates of plain and buffered acetylsalicylic acid (638). The potentiation of injectibles through Al_2O_3 adsorption has been studied (639, 640).

Effect of Particle Size.—The influence of particle size on systemic effects after breathing potent medicated aerosols was investigated (641). Three papers relating griseofulvin absorption to particle size were published: Kraml, *et al.*, studied the effect of particle size and addition of surfactants and corn oil on the serum levels of griseofulvin in rats (642). In another paper, Kraml, *et al.*, found that micronized griseofulvin was absorbed better from the gastrointestinal tract than nonmicronized material (643). The third paper, by Atkinson, *et al.*, discussed the relation of griseofulvin particle size to human blood levels (644). They found that 2.7 μ diam. griseofulvin was twice as effectively absorbed as particles measuring 10 μ in diameter. Rabbit blood levels were compared after administration of metal salts and various particle sizes of chloramphenicol (645). In another study, the effects of particle size and dissolution rate on blood concentration following oral administration of N′-(5-ethyl-1,3,4-thiadiazol-2-yl) sulfanilamide were reported; an instrument for measuring dissolution rate was also described (646). The influence of particle size and solubilizers on the absorption of spironolactone from the gastrointestinal tract was studied (647). Purity was found to be more important than particle size to the anthelmintic efficacy of phenothiazine (648).

Absorption Control.—Several reviews were published on various aspects of prolonged-action medication (649–652). An article on the dangers of using oral sustained-release medication was also published (653). Horstman used a double-blind study to show that an extended-action tablet was more effective than a regular tablet of a given drug (654). Two-layer tablets with prolonged action were investigated (655). Exponential release of cyclobarbital calcium from different oral sustained-action dosage forms in artificial gastric juices was reported (656).

Moeller and Rider evaluated a long-acting anticholinergic-antacid preparation in the treatment of peptic ulcer and other diseases of the digestive tract (657), while Hock described clinical evaluation of an anticholinergic agent in a sustained-release dosage form (658). The relationship between dissolution of sustained-release granules and rabbit blood levels of chloramphenicol was investigated (659). Clinical observations of several diabetic patients taking phenethylbiguanide timed-disintegrating capsules were reviewed (660). Weller, *et al.*, also studied the use of phenethylbiguanide timed-disintegrating capsules in the management of diabetic patients (661). The effect of sustained-release acetazolamide on intraocular pressure in man was investigated (662). Rudolph, *et al.*, discussed the use of multiple miniature long-acting antihistamine tablets in allergy (663), and the clinical and physiological effectiveness of timed-release coronary dilator therapy was investigated by Robbins and Thompson (664). Two reports dealt with the release of iron from oral sustained-action dosage forms (665, 666).

The rate of absorption and duration of plasma level after administration of different pharmaceutical preparations of meprobamate were investigated (667). Hollister compared blood levels from compressed tablets and two delayed-action capsule forms of meprobamate (668). In another study, Hollister evaluated two phenothiazine tranquilizers administered in coated tablet and prolonged-action dosage forms (669). S^{35}-labeled trimeprazine tartrate was used to study the effect of sustained release on absorption and excretion (670). Cass and Frederik discussed clinical evaluation of long-release and capsule forms of pentobarbital sodium (671). Enteric-

coated tablets of prednisolone (672) and an oral sustained-release form of methylprednisolone have been investigated (673). A vitamin B_{12} preparation with retarded absorption was reported by Schwartz, *et al.* (674), and Arnold, *et al.*, found that an oil-aluminum monostearate depot preparation of vitamin B_{12} prolonged levels up to 27 days (675).

A study of iron dosage and absorption demonstrated the existence of a linear relationship between dose and absorption when both are plotted on logarithmic scales (676). Blood and urine levels after oral doses of a new soluble tetracycline have been investigated (677). Atkinson, *et al.*, analyzed human blood levels produced by different dosage schedules of griseofulvin; rate of absorption and blood level appeared to be linearly related to the logarithm of the dose (678). Different dose schedules of amethopterin and the resultant serum and tissue concentrations and urinary excretion patterns were examined (679). The stimulating effects of some inhibitors of drug metabolism on excretion of ascorbic acid and drug metabolism were also studied (680).

Kinetic Studies.—Applications of chemical kinetics to the absorption and excretion of chemicals have been reviewed (681). Taylor and Wiegand have demonstrated the usefulness of an analog computer in analyzing models of biological processes involving plasma-drug kinetics (682). To illustrate, they examined drug-plasma kinetics after sustained-release medication. The use of digital computers for fitting kinetic data to models was reported by Berman, *et al.* (683). Berman, *et al.*, also described some formal approaches to the analysis of kinetic data in terms of linear compartmental systems (684). Another researcher described a method for the determination of absorption rates by integration of rates of appearance and disappearance in blood (685).

An *in vitro* study of the kinetics of penetration of sulfonamides through the intestinal barrier was published (686). Nelson, *et al.*, have studied the influence of tolbutamide absorption rate on the rate of decline of blood sugar levels in normal humans (687), and Nelson discussed the zero-order oxidation of tolbutamide *in vivo* (688). In another paper, Nelson examined the kinetics of excretion of sulfonamides during therapeutic dosage regimens (689). A kinetic investigation of blood sugar lowering by various types of insulin preparations resulted in a method for the determination of rate constants (690).

Schwarz, *et al.*, studied the resorption and biological half-life of glycerin in humans (691). The relationship between plasma salicylate concentration and rate of urinary salicylate excretion was explored (692). Also investigated was the effect of protein binding on the renal excretion rate of salicylic acid derivatives; constants for excretion velocity and half-life were reported (693). Penetration kinetics of basic drugs such as aminopyrine through the intestinal barrier *in vitro* were discussed (694). Garrett, *et al.*, used an analog computer to study the influence of steroids on Ca^{47} dynamics in dogs (695). Kinetic studies were also used for investigating fungicidal action (696) and the activity of pilocarpine and eserine (697). Distribution and excretion kinetics of a new dextran were reported (698). This new dextran was made by direct microbiological conversion of sucrose into a material of appropriate molecular weight. Moll and Code investigated the rates of absorption of sodium and potassium from the stomach and upper small bowel of rats (699, 700). The kinetics of accumulation of radioiodine by the thyroid over both short- and long-time intervals was also investigated (701, 702).

Absorption Mechanisms.—Mechanisms of drug absorption and excretion have been reviewed (703). Hindle and Code reported some differences between duodenal and ileal absorption; the duodenal process appeared directed toward establishment of an equilibrium with blood, while the ileal process tended more toward absorption (704). The relationship between pharmacological antagonsim and membrane potential has been investigated (705). Rectal absorption of antibiotics was also studied (706). Ballard and Nelson reviewed absorption of implanted solid drug (707), and Barr cited over 200 references in a review of percutaneous absorption (708). Barriers to skin penetration were reported in another paper (709). The influence of urinary pH on the excretion and biotransformation of salicylic acid derivatives was investigated (710). Kostenbauder, *et al.*, described methods for controlling urinary pH and the resultant effect on sulfaethidole excretion in humans (711). Other investigators discussed the adjustment of urine pH in chemotherapy of urinary-tract infections (712).

An active transport system for absorption of folic acid from the small intestine of the rat has been reported (713). Amino acid absorption in the rat upper small intestine was found to be an active process dependent, at least in part, on vitamin B_6 (714). Considerable research effort was devoted to mechanisms involved in vitamin B_{12} absorption (715–718). Measurements of

folic acid and D-xylose absorption have been used as tests of small-intestine function (719). Beck, *et al.*, described the use of D-xylose blood level time curves as an index of intestinal absorption (720). In their studies on the mechanism of intestinal absorption of sugars, Bihler and Crane (721), and Bihler, *et al.* (722), investigated the influence of cations on the active transport of sugars. Renal disposal of salicyluric acid has been discussed (723). Schanker and Jeffrey (724) and Schanker and Tocco (725) have reported their work on the pyrimidine transport process of the small intestine. The influence of bioelectric potentials on absorption and distribution of organic electrolytes was investigated (726). Barry, *et al.*, concluded that potential differences arising from ion movements across the gut were not responsible for water transport (727).

Drug Absorption: Antibiotics.—Physical, chemical, and pharmacodynamic studies of viomycin have been reviewed (728). Griffith and Black presented a comparison of blood levels after oral administration of erythromycin and erythromycin estolate (729). They reported that a 1000-mg. oral dose of erythromycin is needed to obtain the same blood levels produced by a 250-mg. oral dose of erythromycin estolate. Serum levels following parenteral administration of paromomycin (730) and modes of excretion and accumulation of water-soluble and basic antibiotics in the mouse (731) have been investigated.

Serum binding, distribution, and excretion of tetracycline and a new analog, methacycline, were compared in humans (732). Scheiner and Altemeier studied the factors inhibiting absorption and effective therapeutic levels of demethylchlortetracycline hydrochloride and found that milk, milk products, and aluminum hydroxide gel inhibited absorption (733). When combined with lysine, tetracycline and chlortetracycline were absorbed into the rat intestine *in vitro* at a faster rate than either antibiotic alone (734). Antibacterial activity, *in vitro* absorption, diffusion, and elimination of tetracycline-L-methylenelysine by man have been investigated (735). Krondl, *et al.*, studied the influence of chlortetracycline and neomycin on fat digestion and absorption in rats (736).

The absorption of some new chloramphenicol esters has been reported (737). In their studies on the absorption and excretion of drugs, Kakemi, *et al.*, measured chloramphenicol blood levels after administration of the free drug or its esters (738). In another paper, they discussed determination of chloramphenicol in urine and its metabolic rate (739). They also studied absorption of chloramphenicol and the effect of metal salts on chloramphenicol absorption from the rat stomach and small intestine (740).

Russell reviewed some of the properties and pharmacological actions of new synthetic penicillins (741). Bacteriological and clinical pharmacological investigations of methicillin and oxacillin have been reported; methicillin was found unstable in acid solution but oxacillin was well absorbed orally from an empty stomach (742). The effect of ascorbic acid on the absorption of potassium phenoxymethylpenicillin was examined (743). Cheng and White studied the effect of orally administered neomycin on the absorption of penicillin V and discovered that neomycin inhibited absorption (744). Chemotherapeutic properties of some α-substituted benzylpenicillins have been described (745). Administration of penicillin decreased the coefficient of intestinal absorption of *p*-aminohippuric acid in man and rat (746).

Drug Absorption: Organic.—Richmond and Girdwood (747) and Christensen (748) have published papers dealing with amino acid absorption. Everted hamster intestinal sacs were used to study amino group requirement for transintestinal transport of amino acids against a concentration gradient (749). Absorption, distribution, and excretion of ϵ-aminocaproic acid following oral or intravenous administration to man were investigated (750). Absorption of amino acid mixtures from the small intestine of the rat was also investigated (751). Equimolar mixtures and mixtures simulating the amino acid composition of egg albumin, casein, and zein were studied. Gastrointestinal absorption and renal excretion of hydroxyproline peptides in humans (752) and glucose, amino acid, and urea absorption from the canine intestine (753) were explored.

Levy and Sahli compared the gastrointestinal absorption of aluminum acetylsalicylate and acetylsalicylic acid (754) and Leonards demonstrated the presence of acetylsalicylic acid in plasma following oral ingestion of aspirin (755). Water and electrolyte pharmacodynamics of aspirin absorption were investigated (756). Tanaka, *et al.*, studied the effect of glucosamine on absorption and excretion of salicylic acid derivatives in rabbits and dogs (757, 758). Absorption of salicylic acid derivatives from the rat stomach and small intestine was investigated by Kakemi, *et al.* (759, 760). In another paper, Kakemi, *et al.*, described a method for the sepa-

ratory determination of human urinary metabolites of *p*-aminosalicylic acid (761). The influence of a nonionic surfactant on percutaneous penetration of diethylamine salicylate was studied (762), and coefficients of intestinal absorption of *p*-aminohippuric acid in man and some laboratory animals were reported (763).

Studies on the digestion, absorption, and metabolism of castor oil were published (764). Absorption, metabolism, and excretion of some edible dyes have been investigated (765, 766). Urinary excretion of tritiated butylated hydroxyanisole and butylated hydroxytoluene in the rat was studied by Golder, *et al.* (767). The absorption of bretylium and related quaternary ammonium salts from the alimentary tract has been investigated (768). Kaul studied the urinary excretion of tetrahydroaminoacridine (769), and Weiner and Lack found that little, if any, absorption of bile salts took place in the proximal portion of the guinea pig intestine (770).

Results of studies on absorption by the guinea pig intestine of cyanocobalamin incubated with intrinsic factor were published (771). Rat utilization of riboflavin and thiamine given orally or parenterally at periodic intervals was investigated (772). Booth and Brian studied the absorption of tritium-labeled pyridoxine hydrochloride in the rat (773). They found pyridoxine to be rapidly absorbed from the upper intestinal tract regardless of the amount administered. Percutaneous absorption of nicotinic acid and ethyl nicotinate through human skin was also investigated (774).

In their studies on the absorption and excretion of medicaments, Kakemi, *et al.*, used ion-exchange resins to separate metabolic products of isoniazide from the urine (775). The absorption of a bronchodilator, isoetharine (776), and absorption and excretion of sulfamethomidine, a "long-acting" sulfonamide (777), have been investigated. Oral absorption of sulfadimethylpyrimidine was studied (778). Gantt, *et al.*, discussed the gastrointestinal absorption of spironolactone (779). Studies on the distribution and excretion of mebrophenhydramine in rats have been reported (780). Absorption, excretion, and fate of prodilidine were studied in the rat by perfusing the small intestine and measuring drug concentration in the effluent (781).

Drug Absorption: Inorganic.—Interrelation of calcium and magnesium absorption was investigated by Alcock and MacIntyre (782). Normal rates of absorption of water, sodium, and potassium in man and animal have been investigated (783). The influence of osmolarity on water and electrolyte flux rates in the duodenum, jejunum, ileum, and colon was explored; net flux of sodium ions and chloride ions in the dog ileum was found to be entirely due to diffusion (784). Edelman described a four-component system for the transfer of water and sodium in blood and tissues (785), and Ginsburg discussed the equilibration of potassium in blood and tissues (786).

Numerous publications appeared on the subject of iron absorption. Cantrill and Walsh (787, 788) and Cantrill, *et al.* (789), investigated iron absorption from the intestinal tract. Brise (790, 791) and Brise and Hallberg (792–795) studied various aspects of iron absorption using radioactive iron-55 or iron-59. Absorption of radioactive iron from segments of the duodenum and ileum of dogs was studied (796). Kinetics of iron absorption in mice has been investigated (797). The effects of oral iron and pregnancy on the active transport of iron by the intestine were reported (798), and Manis and Schachter described a two-step mechanism for the active transport of iron by the intestine (799). Other investigators discussed the role of the duodenum in iron absorption (800). Evidence of serosal "absorption" by the small intestine after intraperitoneal iron injections was presented (801). Results of an investigation by Loria, *et al.*, indicated that concurrent administration of sorbital improved iron absorption in man (802). Ferroglycine sulfate was found to have no advantage over ferrous sulfate (803). Radio-iron was used to study iron absorption following rectal administration (804).

PLANT CHEMISTRY[2]

The volume of literature published in this area during 1962 is tremendous. This is not too surprising since such a small percentage of the world's plants have been investigated from a medicinal point of view. References in this area have been divided into two groups. First is a *Phytochemical Investigation* section which is concerned with studies of specific plants and/or alkaloids; second is a section on *Methodology*.

Phytochemical Investigations.—Several methods of presenting the references for this section were considered. A listing of references according to the plant(s) studied was finally adopted. Such a procedure provides easy reference to the work on a particular plant which may be of interest to the individual phytochemist. Table I, therefore, lists alphabetically each

[2] The writer thanks Dr. G. H. Svoboda for many helpful suggestions concerning the preparation of this section.

TABLE I.—PHYTOCHEMICAL INVESTIGATIONS

Plant	Reference
Acrospira asphodeloides	(805)
Allium sativum	(806)
Amphipterygium adstringens	(807)
Anabasis articulata	(805, 808)
Anabasis setifera	(805)
Angelica edulis	(809, 810)
Arachis hypogaea	(811)
Argemone mexicana	(812)
Aristolochia triangularis	(813)
Artemisia annua	(814)
Artemisia vulgaris	(815)
Aspergillus terreus	(816)
Athyrium mesosorum	(817, 818)
Baccania cordata	(819)
Balanops australiana	(820)
Bersama abyssinica	(821)
Caccinia glauca	(822)
Calophyllum inophyllum	(823)
Casearia sylvestris	(813)
Cassia auriculata	(824)
Cassia goratensis	(824)
Catalpa ovata	(825)
Catha edulis	(826)
Cestrum parqui	(827)
Chenopodium ambrosioides	(828)
Claviceps species	(829)
Cochlospermum gossypium	(830)
Corydalis incisa	(831–834)
Corydalis solida	(835)
Cynanchum caudatum	(836, 837)
Datura species	(838–842)
Daucas carota	(843)
Decodon verticillatus	(844)
Digitalis species	(845–850)
Dryopteris barbigera	(851)
Dryopteris cochleata	(851)
Erysimum diffusum	(852)
Ficus religiosa	(806)
Geissospermum vellosii	(853)
Genista raetam	(854)
Ginkgo biloba	(855, 856)
Ginseng	(857, 858)
Haloxylon salicornicum	(854)
Helenium species	(859, 860)
Leonurus sibiricus	(861, 862)
Leucojum aestivum	(863)
Liriodendron tulipifera	(864)
Litsea japonica	(865)
Lobelia species	(866, 867)
Lopharia papyracea	(868)
Lychnis alba	(869)
Lycopodium species	(870–873)
Magnolia coco	(874)
Magnolia kachirachirai	(874)
Marsdenia tomentosa	(875)
Matricaria chamomilla	(876)
Menispermaceae alkaloids	(877–881)
Metaplexis japonica	(882)
Michelia alba	(883)
Michelia compressa	(884–887)
Mollugo nudicaulis	(888)
Ornithogalum umbellatum	(889)
Orixa japonica	(890)
Papaver species	(891, 892)
Pancratium maritimum	(854)
Pancratium sickenbergeri	(854)
Phegopteris polypodioides	(893)
Pinus species	(894, 895)
Pisum sativum	(896)
Pteryxia terebinthina	(897)
Rauwolfia species	(898, 899)
Rhamnus alaternus	(900)
Rivea corymbosa	(901)
Schinopsis balansae	(813)
Schinus terebinthefolius	(902–904)
Serratia plymuthicum	(905)
Sinapis alba	(906)
Swartzia madagascariensis	(805)
Thalictrum actaefolium	(907)
Thalictrum thunbergii	(908–911)
Tillandsia usneoides	(912)
Tylophora crebriflora	(913)
Veratrum grandiflorum	(914)
Veratrum stamineum	(915)
Vinca major	(916)
Vinca rosea (*Catharanthus roseus*)	(917–920)
Wrightia tinctora	(921)

plant studied, followed by an appropriate reference to the bibliography.

Methodology.—For the purpose of this review, a rather broad interpretation of the subsection title is necessary. While it is intended to be a section primarily concerned with phytochemical methodology, a few references which would not fit conveniently into the preceding sections were included.

A general review of the technology and chemistry of alkaloids was published by Poisson (922). Other investigators studied the preparation of a dry extract of belladonna (923) and the action of boric acid and some of its polyhydroxy complexes on plants (924). Three publications on essential oils discussed such topics as methods of separation, production, composition, and industrial mechanization (925–927). Natural plant hydrocolloids were the subject of three papers by Meer and Meer; origin, physical chemistry, and applications of several gum and gumlike materials were discussed (928–930). Differential studies on plant gums have also been reported (931). Saiki considered the application of statistics to the field of plant internal morphology (932–934). Statistical evaluation of two parameters permitted the classification of Japanese aconites into five types. Two papers dealt with the adsorption of extracted materials on powdered herb residues (935) and the adsorption of ergot alkaloids on activated charcoal (936).

Tissue culture has been successfully used in the field of plant chemistry. Babcock and Carew initiated cultures of callus tissue from several species of *Apocynaceae* (937). In another paper, Staba studied nutritional requirements in tissue cultures of *Digitalis lanata* and *D. purpurea* (938). As part of a study on enzymatic and nonenzymatic oxidation of therapeutically active plant substances, the oxidation of pyrocatechols by peroxydase was investigated (939). Another report dealt with conditions for precipitation of alkaloids

and synthetic bases with Reinecke salt (940). Ali and Guth explored the possibilities of freeze-drying water infusions of alkaloid-containing drug plants (941).

Drug extraction with isopropyl and ethyl alcohols has been reviewed (942). One investigator described an enzymatic procedure for the extraction of alkaloids from *Rauwolfia serpentina* (943), while another studied the dynamics of extraction of rutin from *Sophora japonica* (944). Better extraction and less solvent loss resulted from the application of centrifugal force to the extraction of plant materials (945). Kubiak explored the effect of ultrasonics on the quantitative and qualitative results of plant extraction (946). The ultrasonic energy appeared to increase the extraction of active materials with no decomposition. The action of ultrasound on alkaloids has also been investigated (947).

Several papers were published in the past year on the use of chromatography in phytochemical studies. Farnsworth and Euler reported an alkaloid-screening procedure utilizing thin-layer chromatography (948). Thin-layer chromatography was also used for separation of the active principles of *Ammi visnaga* and *Ammi majus* (949). Sjoholm employed a two-dimensional thin-layer chromatography technique for studying some digitalis glycosides (950). A new solvent system was recommended for the paper chromatography of some veratrum alkaloids and their derivatives (951). Ascending chromatography on a hydrophobic paper was used in a study of the constituents of *Cannabis sativa* (952), and Meer, *et al.*, reported a rapid method which utilizes an ascending paper chromatography technique for the identification and evaluation of botanical extracts (953). Other investigators discussed chromatographic evaluation of glycoalkaloids in *Solanum aviculare* (954). Paper chromatography was also used in a study of the hydrolysis of some alkaloid esters; R_f values for several alkaloids and their hydrolysis products were reported (955).

EQUIPMENT

Automation in the pharmaceutical industry was the subject of several papers published last year. One reported on the automated production of aspirin (956), while two others discussed automation in antibiotic and vitamin manufacturing (957, 958). Automatic coating of tablets was studied (959), and Gabrysh, *et al.*, described an automatic rotational viscometer and high pressure apparatus for the study of non-Newtonian systems (960). Dunlop discussed the mechanization of tablet and capsule packaging (961). In another paper, Schroeter, *et al.*, presented an apparatus for automated dissolution rate studies of capsules and tablets (962).

A review of grinding and pulverizing equipment was published (963). In a series on small-scale processing machinery, Fowler discussed mixers for powders and semisolids (964, 965). A flow-ultramicroscopic method of determining the number concentration and particle-size analysis of aerosols and hydrosols was described (966). Another group of investigators also reported a particle-size analyzer for aerosols (967). An electronic-microscopic method for precise size measurement and analysis of particulate pharmaceutical materials was published by Barnett and Timbrell (968).

Recent advances in freeze-drying were reviewed by Greaves (969). The application of spray-drying techniques to the production of anti-anemia concentrates was also reported (970). Muller discussed rotation viscometers and their application to non-Newtonian measurements (971), and Fairgrieve developed a thermostated bath for viscometry (972). A modified dual-unit organ bath for isolated tissues was also described (973). Continuous recording methods for measuring perspiration were reported by O'Malley (974) and Marchisotto (975).

An apparatus for testing the resistance to wet heat of bacterial spores on paper carriers was described (976). Preparation of sterile ophthalmic solutions in the pharmacy was the subject of an article by Putney (977). A small-scale sterilization apparatus was made by an adaptation of a 5-ml. syringe (978). Three papers were published on the properties of filter materials (979–981). Wolffgang described a simple apparatus for rapid dialysis of small samples (982), and Hart presented some general considerations to aid in centrifuge selection (983). A comparison of Bittel and Cornish systems for separation of stereoisomers by countercurrent distribution was also published (984).

CONCLUSION

The scope of the literature covered in this review is an indication of the complexity of pharmaceutical research today. As pharmaceutical research becomes even more complex in the years ahead, the pharmaceutical scientist will be increasingly dependent on developments in other disciplines. An up-to-date knowledge of the literature provides a sound basis for interdisciplinary communication.

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[3] *Index Medicus* abbreviations were used for journals not listed in *Chemical Abstracts*. In a few instances where the writer was unable to obtain late 1962 issues, references were obtained from *Current Contents*.

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Gastrointestinal Absorption of Aspirin Anhydride

By GERHARD LEVY and BERNICE A. GAGLIARDI

The gastrointestinal absorption of aspirin anhydride by humans has been found to be slow and incomplete. This explains and is consistent with a reported lack of therapeutic efficacy of this drug. It is concluded that aspirin anhydride is less satisfactory for salicylate therapy than aspirin itself.

CONTINUOUS ATTEMPTS have been made to develop new derivatives and dosage forms of salicylic acid having greater effectiveness, better absorption characteristics, and less tendency to cause gastrointestinal irritation and bleeding than presently used salicylate preparations. It has been suggested (1) that (based on physical-chemical evidence) aspirin anhydride should be a superior form for the oral administration of aspirin. While aspirin anhydride has been studied recently with respect to its tendency to cause gastrointestinal bleeding (2, 3), no information concerning the gastrointestinal absorption and physiological availability of this substance has yet appeared in the literature.

There has been particular reason to question the absorption efficacy of aspirin anhydride since its solubility in aqueous media is extremely low, namely 3.2 mg. per 100 ml. at 37.5° (1), which is less than one-hundredth the solubility of undissociated aspirin itself. Another poorly soluble and slow-dissolving salicylate, aluminum acetylsalicylate, recently has been found to be absorbed not only very slowly but even incompletely (4). In the present communication the results of our absorption studies with aspirin anhydride are reported and related to available pharmacologic and physical-chemical data.

EXPERIMENTAL

Materials.—Micronized acetylsalicylic acid U.S.P. and micronized aspirin anhydride were utilized. Both compounds were assayed colorimetrically in terms of salicylic acid (9) after alkaline hydrolysis. One gram of acetylsalicylic acid was found to be equivalent to 0.96 Gm. aspirin anhydride (theoretical: 0.95 Gm.) on the basis of the salicylic acid assay. The drugs were handfilled into hard gelatin capsules, size 00, which were weighed individually before and after filling to obtain exact dosage. The specific surface area of each drug was determined by the nitrogen adsorption method and was found to be 1.22 M.²/Gm. for micronized aspirin and 2.24 M.²/Gm. for micronized aspirin anhydride.[1]

Absorption Tests.—Male adults in apparent good health served as test subjects. Their weights and ages are listed in Tables I and II. In the study of early absorption, each subject received two capsules containing a total of either 1 Gm. aspirin or 0.96 Gm. aspirin anhydride about 1 hour after a light noon meal. The medication was administered together with 100 ml. of water, and another 100 ml. water was given exactly 1 hour later. The two drugs were administered in crossover fashion, 1 week apart. One-half the group received aspirin first, while the other half received aspirin anhydride first. All subjects were instructed to observe similar dietary habits on the two days scheduled for the studies. A small urine sample was collected immediately prior to drug administration to obtain blank values and to make certain that the subjects had not inadvertently ingested a salicylate-containing medication during the 24 hours preceding the test. Exactly 2 hours after drug administration, total urine was collected from each subject.

The total absorption study was initiated in the morning after an overnight fast. The subjects were instructed to empty their bladder of overnight urine and to drink one glass of water. One hour later, after collection of a small urine sample, either 1 Gm. of aspirin or aspirin anhydride in two hard gelatin capsules was administered, together with 100 ml. of water. Total urine was collected exactly 2 hours after drug administration and from then on at 2–3 hour intervals (except for a greater night-time interval) for at least 36 hours. The subjects did not eat until 1 hour after drug administration.

The urinary excretion method for measuring salicylate absorption has been employed previously in our laboratory (4, 5) and by other workers cited in a previous publication (6). Urinary salicylate excretion data correlate well with plasma salicylate levels (5) as can be expected, although excretion data are possibly more accurate since they represent cumulative rather than transitory values.

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[1] We thank Mr. E. L. Rowe, Pharmacy Research Section, Product Research and Development, The Upjohn Co., for the surface area measurements.

TABLE I.—URINARY EXCRETION OF APPARENT SALICYLATE[a] 2 HOURS AFTER ORAL ADMINISTRATION OF EQUIVALENT DOSES[b] OF ASPIRIN AND ASPIRIN ANHYDRIDE

Subject	Age, yrs.	Wt., lbs.	Aspirin Amount, mg.	Aspirin Urine Vol., ml.	Aspirin Urine pH	Aspirin Anhydride Amount, mg.	Aspirin Anhydride Urine Vol. ml.	Aspirin Anhydride Urine pH	Ratio[c] ASA/AA
N	21	130	49.8	107	7.0	16.7	267	6.4	3.0
B	21	160	6.9	168	5.6	2.8	185	6.9	2.5
C	24	177	16.2	106	6.6	8.3	74	5.9	1.9
S	22	155	39.3	59	5.6	17.9	211	5.5	2.2
Go	22	150	14.6	31	5.6	7.3	64	5.6	2.0
F	21	185	7.6	153	5.6	10.1	206	5.2	0.75
Cl	21	125	25.4	64	5.6	12.5	69	5.5	2.0
A	23	173	36.8	55	5.7	8.4	67	5.7	4.4
Average			24.6	93	5.7	10.5	143	5.6	2.3

[a] Expressed as salicylic acid. [b] 1 Gm. aspirin and 0.96 Gm. aspirin anhydride. [c] ASA = mg. salicylate excreted after administration of aspirin. AA = mg. salicylate excreted after administration of aspirin anhydride.

TABLE II.—SECOND-HOUR AND TOTAL URINARY EXCRETION OF APPARENT SALICYLATE[a] AFTER ORAL ADMINISTRATION OF 1 GM. ASPIRIN

Subject	Age, yrs.	Wt., lbs.	Amount Excreted After 2 Hr., mg.	Total Amount Excreted, mg.	Total Urine Vol., ml.	Av. Urine pH	Total Amount Excreted in Previous Test
L	33	170	73.2	555	1340	5.9	538
R	21	190	59.5	683	1830	5.9	...
G	22	200	27.9	374	3420	5.5	399
Average			53.5	537	2197	5.7	

[a] Expressed as salicylic acid.

Urine pH.—The pH of each urine sample was determined with a Leeds and Northrup pH meter, model 7664. The salicylate excretion rate increases rapidly when urinary pH exceeds 7.0 (7, 8), and an unusually high urinary pH during one test period but not during the other could distort significantly the comparative data. These considerations caused the elimination of one subject participating in the early absorption test since the pH of his urine exceeded 7.0 in one instance.[2]

Analytical Methods.—Salicylate in the urine was determined colorimetrically according to Trinder (9) with a Bausch and Lomb Spectronic 20 colorimeter. All readings were corrected for blank values. Zero-hour blank values were used for second-hour urine samples, and average blank values obtained from previous 24-hour urine collections were used in the assay of all other samples.

RESULTS AND DISCUSSION

Table I shows the results of the study of early absorption. On the basis of the amounts of salicylate excreted 2 hours after drug administration, it is apparent that aspirin anhydride is absorbed considerably less rapidly than aspirin. The difference in amounts of excreted salicylate is statistically significant ($p < 0.05$), although there is considerable variation within each group. This is quite usual since our previous salicylate absorption studies and those of others indicate that subjects can be classified on a fairly consistent basis as either rapid, average, or slow absorbers.[3] This is evidenced in the present study by the much more consistent results obtained when the data are expressed as a ratio of salicylate excreted from aspirin to salicylate excreted from aspirin anhydride (see Table I). As shown in the tabulation, average urinary pH was essentially the same in both groups. Since the pH scale is nonlinear, and therefore nonadditive as such, the individual pH values were first converted to molar terms, then averaged, and finally the average molar concentration was reconverted to pH units. There may be some question as to the propriety of averaging pH values even in this manner, but it was done here as an aid in summarizing the data.

Urine flow rate has been found to have a small though noticeable effect on salicylate excretion (8, 10). It is unlikely that the small differences in urine flow noted in the present study had a significant effect on salicylate excretion, but even if they did, it could only be in favor of the aspirin anhydride results.

Results of the total absorption study are listed in Table II for aspirin and in Table III for aspirin anhydride. Average urinary pH and volume did not differ significantly after administration of the two substances. Since the subjects who participated in this study took the drugs on empty stomachs, absorption as evidenced by second-hour salicylate excretion was faster than in the other group of subjects who took the drug after a light meal. The average ratio of salicylate excreted from aspirin to salicylate excreted from aspirin anhydride was, however, essentially the same in both groups, namely 2.3 and 2.2. The total amount of apparent salicylate excreted after aspirin anhydride administration was considerably less than after aspirin administration. This shows that aspirin anhydride is not absorbed completely. It

[2] Similar precautions are appropriate in the case of blood level studies, since drug concentrations in the blood are a function of both absorption and elimination rates.

[3] This phenomenon is apparently related to differences in gastric emptying rates.

TABLE III.—SECOND-HOUR AND TOTAL URINARY EXCRETION OF APPARENT SALICYLATE[a] AFTER ORAL ADMINISTRATION OF 1 GM. ASPIRIN ANHYDRIDE

Subject[b]	Amount Excreted After 2 Hr., mg.	Total Amount Excreted, mg.	Total Urine Vol., ml.	Av. Urine pH	Physiologic Availability, %[c]
L	36.4	412	2440	6.1	71
R	14.7	428	3910	5.7	60
G	22.9	184	2315	5.8	47
Average	24.7	341	2888	5.8	61

[a] Expressed as salicylic acid. [b] Ages and weights are listed in Table II. [c] Calculated according to a method described in the text.

has been established previously that the effect of absorption rate on the ratio of the principal metabolites of salicylic acid appearing in the urine is negligible at the dosage used in this study (4). Of interest is the fact that the total amount of apparent salicylate excreted after a given dose differed significantly among the subjects. This is apparently due to quantitative differences in the biotransformation of salicylate, causing different degrees of formation of metabolites not identifiable by the colorimetric method. These differences reflect the biochemical individuality of each subject. Total excretion data from a previous aspirin absorption study were available for two subjects and are listed in Table II. It can be seen that the values are very similar to those obtained in the present study, suggesting that these individual biotransformation patterns are quite stable.

The physiologic availability of aspirin anhydride was calculated according to the method used in the human bioassay procedure of Melnick, *et al.* (11), which has been applied previously to salicylates by Morrison and Campbell (12) and by the authors (4). Thus, upon administration of equal doses, by weight, of the two salicylates

$$\% \text{ physiologic availability} = \frac{\text{amount excreted after aspirin anhydride}}{\text{amount excreted after aspirin}} \times 100 \times 0.96,$$

the latter term being a correction factor for the slightly higher salicylate content of aspirin anhydride. The data in Table III show that physiologic availability ranged from 47 to 71%, indicating that a significant portion of orally administered aspirin anhydride was not absorbed. It should be pointed out that the drug was used in this study in a physical form highly favorable for absorption, namely as the micronized powder, and that the physiologic availability may be even less when aspirin anhydride is administered in the form of compressed tablets containing drug solids of conventional particle size. The particle size of the aspirin anhydride used in this study was considerably smaller than that of aspirin. Had the specific surface areas of the two drugs been the same, it is likely that the absorption rate of aspirin anhydride would have compared even less favorably with that of aspirin.

Our findings explain the observation by Wood, *et al.* (2), that, in a significant portion of their patients, aspirin anhydride did not produce such satisfactory relief of symptoms as did aspirin. Our data, indicating poor and incomplete gastrointestinal absorption of aspirin anhydride, are also consistent with the toxicologic data reported by Gray, *et al.* (13). These workers found that the LD_{50} of aspirin anhydride in rats was about 50% higher than the LD_{50} of aspirin. Furthermore, death after administration of aspirin anhydride occurred much later than after administration of aspirin.

The gastrointestinal absorption of salicylates, when administered in solid form, is rate-limited by the dissolution process (6, 14), and the slow and incomplete gastrointestinal absorption of aspirin anhydride can be attributed to its extremely low dissolution rate in aqueous media (15). This factor was not taken into account in earlier work by others (1). Moreover, it appears in the light of present knowledge that the theoretical calculations presented in the early work (1) resulted in an underestimation of the absorption rate of aspirin from solution, partly because absorption of this drug from the stomach (16, 17) was neglected in the calculations, but primarily because the absorption rate of aspirin was assumed to change with pH of the gastrointestinal fluids exactly according to theory. Subsequently, it has been established, however, that the decrease in absorption rate of weak acids with increasing pH of intestinal fluids is much less than predicted by the Henderson-Hasselbalch equation, because the pH directly at the site of absorption remains lower than that of the intestinal fluids (18).

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Synthesis of Some γ-Substituted α-Amino γ-Lactones

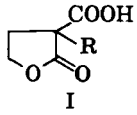
By METRO FEDORCHUK† and FRED T. SEMENIUK

γ-Substituted α-amino γ-lactones were synthesized as potential antibacterial agents. A series of new γ-substituted α-aceto and α-oximino γ-lactones is reported.

THE OCCURRENCE of the lactone ring in many physiologically active compounds has been reported throughout the literature. Of interest here are the γ- and δ-lactones with antibacterial activity.

The mechanisms by which these antibacterials exert their activity differ from one series to the next. Nevertheless, the presence of the lactone structure cannot be ignored. Patulin (1), crepin (2), the active principles of *Spiraea aruncus* (2), and *Arctium minus* (3), for example, lose their antibacterial activity after their lactone moieties are broken. Similarly the macrolide, etamycin (4), is inactivated on hydrolysis of the lactone ring. Geiger and Conn (5) theorized that, since both penicillic acid and patulin are inactivated by an excess of thiol, their antibacterial activities are probably due to the addition of the —SH groups of bacterial enzyme systems or the —SH groups of essential metabolites with the double bond of the α-unsaturated lactones. They were able to show that α,β-unsaturated ketones closely resembled patulin and penicillic acid, both in their reactivity toward thiols and in their bacteriostatic and fungistatic properties. Cavallito and Haskell (6) pointed out that these reactions appear to proceed by addition of the thiol group to the double bond, followed (in the case of the β-angelicalactone and cysteine or homocysteine) by reaction of the lactone with the amino group and loss of water. Cavallito, *et al.* (7), indicated that a series of lactone aliphatic acids of type I showed a close parallelism in the ability to inhibit growth and in their power to lower surface tension. Asano, *et al.* (8), suggested that the lactones of the γ-hydroxy fatty acids prepared by them inhibited the growth of avian tubercle bacilli through antagonism of the many fatty acids that make up the cell wall.

Since the simple lactone structure appears to confer possible antibacterial activity, the authors have chosen for their study a series of γ-substituted α-amino γ-lactones of type II. These are cyclized γ-hydroxy amino acids. It has been previously found (9) that the exchange of hydrogen atoms for hydroxyl groups in some amino acids converts them into antimetabolites. Many antibiotics (10, 11) derived from bacteria and fungi are amino acid derivatives, peptides, or polypeptides. These naturally occurring antibiotics frequently contain amino acids not found in ordinary proteins. Hence, the authors have modified the simple γ-lactone structure to α-amino acid analogs by varying R and R′ in II, providing a series of new γ-lactones as possible antibacterial agents.



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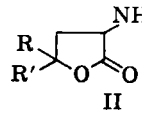

II

Based upon analogy to published results of previous researches, a plausible route for the synthesis of a series of α-amino γ-lactones would have been through the interaction of the epoxide and diethyl acetamidomalonate as illustrated in Fig. 1. In such a manner Sudo, *et al.* (12), prepared α-acetamido-γ-butyrolactone in a 100% yield. Beasley, *et al.* (13), reacted diethyl acetamidomalonate with 1,2-epoxy-3-*o*-toloxypropane, hydrolyzed the resultant α-acetamido γ-lactone, and obtained the α-amino-δ-*o*-toloxy-γ-valerolactone hydrochloride in a 15.2% yield. Sudo, *et al.* (12), used ethanolic sodium ethoxide as a condensing medium, while Beasley, *et al.* (13), used methanolic sodium methoxide as a medium. Dakin (14) was unsuccessful in using ethanolic sodium ethoxide in condensing isobutylene oxide with diethyl acetamidomalonate, but by the use of dry sodium methoxide (with or without dioxane as a solvent) he obtained the desired product in a 50% yield.

In the initial part of this program the authors attempted to condense styrene oxide with diethyl acetamidomalonate in an ethanolic sodium ethoxide medium at room temperature, and at reflux temperature. In both cases attempts failed to isolate the α-acetamido-γ-phenyl-γ-butyrolactone, or the corresponding hydrochloride. When the reaction was carried out at room temperature, 44.2% of the styrene oxide was recovered as the β-ethoxy-α-phenylethyl alcohol resulting from the reaction of sodium ethoxide with styrene oxide; 31.5% of the diethyl acetamidomalonate was also recovered. When the reaction mixture was refluxed, the yield of β-ethoxy-α-phenylethyl alcohol was 56% and only 6% of the diethyl acetamidomalonate was recovered. In the latter case an additional 32.5% of the diethyl acetamidomalonate was recovered as glycine hydrochloride when the residue, assumed to contain the α-acetamido-γ-phenyl-γ-butyrolactone, was hydrolyzed with concentrated hydrochloric acid. Dakin (14) and Beasley, *et al.* (13), also found

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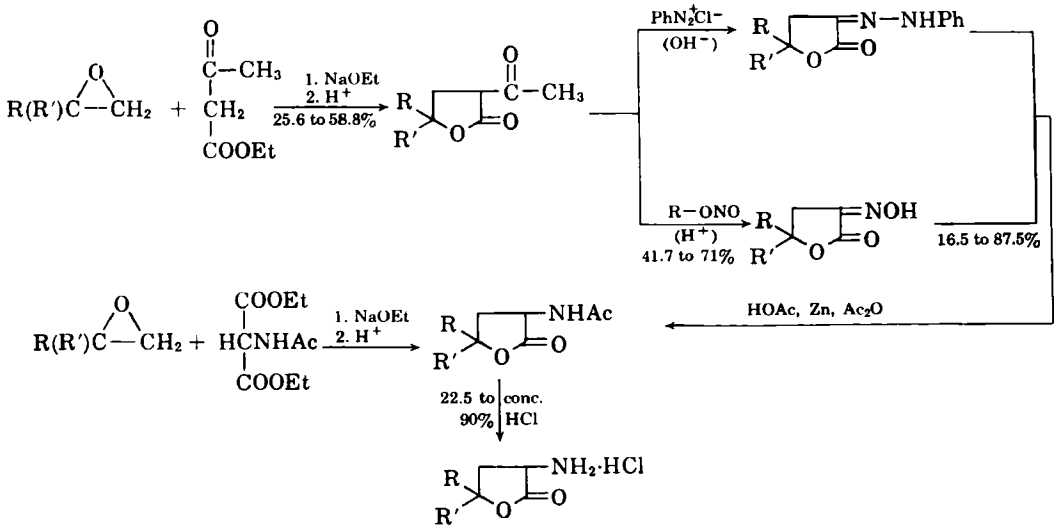


Fig. 1.—Pathways for γ-substituted α-amino γ-lactone syntheses.

that glycine hydrochloride (from hydrolysis of the unreacted diethyl acetamidomalonate) contaminated their final products, presenting some difficulty in purification.

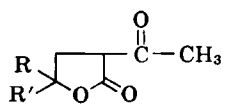
The condensation of propylene oxide with diethyl acetamidomalonate in an ethanolic sodium ethoxide medium at room temperature yielded 3.3% of α-amino-γ-valerolactone hydrochloride on hydrolysis of the corresponding amide—36.6% of the diethyl acetamidomalonate was recovered.

In view of the apparent difficulty to reproduce satisfactorily the published success of others in condensing an epoxide with diethyl acetamidomalonate in an ethanolic sodium ethoxide medium and of the difficulty of separating the unreacted diethyl acetamidomalonate from any product formed, another route for the synthesis of γ-substituted α-amino γ-lactones was sought.

A feasible method for the preparation of these γ-lactones was found in the interaction of the epoxide and ethyl acetoacetate as illustrated in Fig. 1. Four of the α-oximino γ-lactones were not isolated. These were the γ-ethyl-, γ-butyl-γ-ethyl-, and γ-phenyl-substituted α-oximino-γ-butyrolactones, and α-oximino-δ-hydroxy-γ-valerolactone. Attempts to crystallize these oximes from the reaction mixtures failed. Since oximes are known to decompose violently when subjected to elevated temperatures no attempt was made to isolate them by distillation. Consequently the reaction mixtures containing these oximes were subjected to reductive acetylations and the α-acetamido γ-lactones were isolated in all cases except the α-acetamido-δ-hydroxy-γ-valerolactone. In the latter case, the reaction mixture was treated with concentrated hydrochloric acid and the corresponding α-amino-δ-hydroxy-γ-valerolactone hydrochloride isolated.

The yields at the various steps are indicated in Fig. 1 for all cases where the compounds were isolated. α-Acetamido-δ,δ-diethoxy-γ-valerolactone

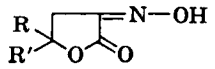
TABLE I.—α-ACETO γ-LACTONES



R	R′	Boiling Range, °C./mm. Hg	Refr. Index/Temp., °C.	Yield, %	Analyses[a] C, % Calcd.	C, % Found	H, % Calcd.	H, % Found
CH_3—	H—[b]	84–86/2.0	1.4525/25	58.8	...	...	...	...
CH_3CH_2—	H—	98–103/0.3	1.4548/26.5	34.7	61.52	61.35	7.75	7.98
$CH_3(CH_2)_4$—	H—	114–118/40	1.4551/28	42.3	66.62	64.94	9.15	8.92
$CH_3(CH_2)_5$—	H—	125–130/1.0	1.4535/28.5	55.5	67.89	67.76	9.50	9.55
$CH_3(CH_2)_7$—	H—	143–150/1.0	1.4570/28	36.4	69.96	69.94	10.07	10.25
C_6H_5—	H—[b]	138–145/2.0	1.5395/25	46.0	...	...	...	...
p-CH_3O—C_6H_4—CH_2—	H—	175–180/0.2	1.5345/22.5	44	...	...	...	...
C_6H_5—O—CH_2—	H—[c]	185–190/1.0	1.5310/27	47.5	...	...	...	...
CH_3—$(CH_2)_3$—	CH_3CH_2—	120–130/2.5	1.4635/24	25.6	...	...	...	...
HO—CH_2—	H—	155–160/0.33	1.4805/26.8	47.5	53.16	53.16	6.37	6.45
$(C_2H_5O)_2CH$—	H—	122–125/0.15	1.4525/25	58.3	...	...	...	...

[a] Analyses by Weiler and Strauss, Oxford, England. [b] Previously reported (15). [c] Previously reported (16).

TABLE II.—α-OXIMINO γ-LACTONES



R	R′	M.p., °C.	Purification Solvent	Yield, %	Analyses[a] C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	N, % Calcd.	N, % Found
CH_3—	H—	106–107	Benzene	71.0	46.53	46.59	5.47	5.55	10.85	10.85
$CH_3(CH_2)_4$—	H—	102–103	Pet. ether (b.p. 60–90°)	59.0	58.35	58.79	8.16	8.08	7.56	8.09
$CH_3(CH_2)_5$—	H—	104–106	Pet. ether (b.p. 60–90°)	63.0	60.26	60.23	8.60	8.17	7.03	7.27
$CH_3(CH_2)_7$—	H—	103–104	Pet. ether (b.p. 60–90°)	67.5	63.40	63.50	9.31	9.18	6.16	6.13
p-CH_3O—C_6H_4—CH_2—	H—	138–140	Benzene	71.0	61.27	61.55	5.57	5.09	...	...
$C_6H_5OCH_2$—	H—	129–131	Benzene-ethanol	45.5	59.72	60.09	5.01	4.87	...	...
$(C_2H_5O)_2CH$—	H—	100–102	Benzene-Pet. ether (b.p. 60–90°)	41.7	49.76	49.86	6.96	7.18	6.45	6.51

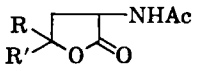
[a] Analyses by Weiler and Strauss, Oxford, England.

was obtained in a 16.5% yield by the reductive acetylation of the corresponding oxime. The other six α-acetamido γ-lactones similarly prepared were obtained in 73 to 87.5% yields.

The reductive acetylation of α-keto γ-lactone phenylhydrazones was also studied as a possible method for the preparation of α-amino γ-lactones. In this study phenylhydrazones of α-keto-γ-valerolactone and α-keto-γ-phenyl-γ-butyrolactone were prepared by the Japp-Klingemann reaction from α-aceto-γ-valerolactone and α-aceto-γ-phenyl-γ-butyrolactone, respectively. The phenylhydrazones were obtained in yields comparable to the yields of oximes obtained from nitrosation reactions. However, upon reductive acetylation of these phenylhydrazones, the yields of the resulting α-acetamido γ-lactones were lower than those obtained when the corresponding oximes were similarly reduced. The most significant disadvantage of this method was the difficulty in separating the acetanilide, formed as a by-product, from the reaction mixture and the product.

α-Amino-γ-phenyl-γ-butyrolactone hydrochloride, α-acetamido-γ-valerolactone, α-amino-γ-valerolactone hydrochloride, and α-amino-γ-pentyl-γ-butyrolactone hydrochloride were screened for biological

TABLE III.—α-ACETAMIDO γ-LACTONES



R	R′	M.p., °C. (b.p., °C/mm. Hg)	Yield, %	Analyses[a] C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	N, % Calcd.	N, % Found
CH_3—	H—	(140–155/20) n^{27} = 1.4820	74.5	53.49	53.65	7.05	7.09	8.91	8.65
CH_3CH_2—	H—	(150–155/0.33) $n_D^{26.5}$ = 1.4782	79.0	56.13	56.63	7.65	7.95	8.18	7.88
$CH_3(CH_2)_4$—	H—	(175–181/1.0) n_D^{27} = 1.4743	81.5	...	...	...	...	6.57	6.96
$CH_3(CH_2)_5$—	H—	67–68.5 from benzene-pet. ether	80.0	63.41	63.70	9.31	9.32	...	...
$CH_3(CH_2)_7$—	H—	59–61 from benzene-pet. ether	80.5	65.86	66.25	9.87	10.04	5.49	4.91
C_6H_5—	H—	159–161 from benzene	10.0	65.75	66.19	5.98	5.86	6.39	6.25
p-CH_3O—C_6H_4—CH_2—	H—	125–127 from benzene-pet. ether	73.0	63.86	64.45	6.51	6.82	...	...
C_6H_5—O—CH_2—	H—	121–123 from benzene-ethanol	87.5	62.64	62.98	6.07	6.58	...	...
$CH_3(CH_2)_7$—	CH_3CH_2—	(167–177/0.5)	58.5	...	...	...	...	...	...
$(C_2H_5O)_2CH$—	H—	108–110 from benzene-pet. ether	16.7	53.86	54.37	7.81	8.08	...	...

[a] Analyses by Weiler and Strauss, Oxford, England.

TABLE IV.—α-AMINO γ-LACTONE HYDROCHLORIDES

R	R′	M.p., °C. (Purification Solvent)	Yield, %	Analyses[a] C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	N, % Calcd.	N, % Found	Cl, % Calcd.	Cl, % Found
CH_3—	H—[b]	200–201 (ethanol)	47.0	...	...	...	7.12	...	7.95	...	...
CH_3CH_2—	H—	203–205 (ethanol)	74.5	43.51	43.53	7.30	8.71	8.46	6.92	17.07	17.22
$CH_3(CH_2)_4$—	H—	131–132 (ethanol-ether)	85.5	52.04	52.52	8.73	8.78	6.74	5.94	...	...
$CH_3(CH_2)_5$—	H—	135–136 (ethanol)	72.0	54.17	54.14	9.09	9.85	6.32	...	...	...
$CH_3(CH_2)_7$—	H—	125–127 (ethanol)	35.0	57.69	58.00	9.68	5.77	5.56	6.45	16.59	16.60
C_6H_5—	H—	223–224 dec. (ethanol-water-ether)	58.0	56.19	56.51	5.66					
p-CH_3O—C_6H_4—CH_2—	H—	201–203 dec. (ethanol)	22.5	55.92	56.59	6.26	6.44	5.44	6.37	...	...
C_6H_5—O—CH_2—	H—	222 dec. (ethanol)	90.0	54.21	53.96	5.79	5.45	5.75	5.40	...	...
CH_3CH_2—	CH_3CH_2—	140–143 (ethanol-ether)	20.5	54.17	51.97	9.09	9.53	6.32	6.33	...	...
HO—CH_2—	H—	213 dec. (ethanol)	19.0	35.83	36.24	6.02	5.89	8.36	8.32	...	...

[a] Analyses by Weiler and Strauss, Oxford, England. [b] Previously reported (17).

activity.[1] The two latter compounds showed some antibacterial activity.

EXPERIMENTAL

The chemical procedures used for the preparation of the various intermediates and products are illustrated by examples which follow.

Epoxides.—1,2-Epoxyoctane, 1,2-epoxydecane, 1,2-epoxy-2-ethylhexane, and 1,2-epoxy-3-*p*-methoxyphenylpropane were prepared according to the method described in "Organic Synthesis" (18) for preparing styrene oxide from styrene. Glycidaldehyde diethyl acetal was prepared according to the method described for preparing acrolein diethyl acetal from acrolein (19).[2]

α-Aceto γ-Lactones (see Table I).—The procedure is essentially that used by Adams and VanderWerf (15) for preparing α-aceto-γ-valerolactone, modified with features used by Van Zyl and Van Tamelen (20) for preparing α-carbethoxy-γ-butyrolactones.

To a cooled solution of 23.0 Gm. (1.0 mole) of sodium in 400 ml. of absolute ethanol (prepared in a three-necked flask equipped with a sealed mechanical stirrer and a reflux condenser fitted with a drying tube), 130.2 Gm. (1.0 mole) of ethyl acetoacetate was added rapidly. The mixture was again cooled in an ice bath and 58.1 Gm. (1.0 mole) of propylene oxide was added dropwise with stirring over a period of 30 minutes. While being stirred, the mixture was kept in the ice bath for several hours and then allowed to come to room temperature. Stirring at room temperature was continued overnight. After cooling the resulting yellow-orange solution in an ice bath to 10°, 60.0 Gm. (1.0 mole) of glacial acetic acid was added; a slurry of sodium acetate formed immediately. The ethanol was removed under reduced pressure, the temperature never being permitted to rise above 50°. Sufficient ice water was added to dissolve the sodium acetate and to separate the resulting oily layer. The aqueous layer was extracted with several portions of ether and the combined crude lactone and extracts were dried overnight over anhydrous magnesium sulfate. The ether was removed and the residue was distilled from a modified Claisen flask equipped with a 15-cm. Vigreux column. Yield was 83.5 Gm. (58.8%) of a colorless oil, b.p.$_2$ = 84–86°, n_D^{25} = 1.4525; reported (15) b.p.$_2$ = 88–90°, n_D^{25} = 1.4489.

α-Oximino γ-Lactones (see Table II).—All of the oximes were prepared by the procedure described for α-oximino-γ-valerolactone: a stream of dry hydrogen chloride gas was introduced for 30 seconds into 60 ml. of cold (0 to −5°) absolute methanol in a three-necked flask, fitted with a mechanical stirrer and thermometer, protected with a drying tube, and surrounded by a freezing mixture. To the methanolic hydrogen chloride solution was added 14.2 Gm. (0.1 mole) of α-aceto-γ-valerolactone. To the cold (0 to −5°) mixture 9.4 Gm. of *n*-propyl nitrite was added dropwise, with stirring, at such a

[1] The authors thank Mead Johnson and Co. for having gratuitously screened these compounds for biological activity.

[2] A sample of glycidaldehyde was provided by the Shell Development Co. 1,2-Butylene oxide was provided by The Dow Chemical Co., glycidol by Roberts Chemicals, Inc.

rate that the temperature did not rise above 0°. On addition of the *n*-propyl nitrite the reaction mixture immediately turned blue. The mixture was maintained at 0° for several hours and was then placed in a refrigerator (5°) overnight. At this point the reaction mixture had assumed a pale yellow color. The methanol and excess *n*-propyl nitrite were removed under reduced pressure with the aid of moderate heat from a water bath. The resulting yellow oil crystallized into a solid mass. This was recrystallized from benzene to give 9.1 Gm. (71.0%) of α-oximino-γ-valerolactone. A sample for analysis, three times recrystallized from benzene, melted at 106–107°.

α-Acetamido γ-Lactones (see Table III).—The procedure used by Fillman and Albertson (21) for the preparation of α-acetamido-γ-butyrolactone was followed as described for α-acetamido-γ-valerolactone. To a solution of 12.9 Gm. (0.1 mole) of α-oximino-γ-valerolactone in 250 ml. of glacial acetic acid and 100 ml. of acetic anhydride 30 Gm. of zinc dust was added portionwise while stirring. The zinc was added at such a rate as to maintain the temperature of the reaction mixture of 50°. After addition of the zinc the temperature was maintained at 50° by applying heat. At the end of 9 hours the warm reaction mixture was filtered. The filtrate was concentrated under reduced pressure and the product distilled at 140–155° at 2 mm. of Hg to give 11.7 Gm. (74.5%) of a clear, slightly yellow, viscid oil, $n_D^{27} = 1.4820$.

α-Amino γ-Lactone Hydrochlorides (see Table IV).—All of the hydrochlorides were prepared by the procedure described for α-amino-γ-valerolactone hydrochloride. A mixture of 5.88 Gm. (0.0375 mole) of α-acetamido-γ-valerolactone and 40 ml. of concentrated hydrochloric acid was refluxed for 5.5 hours. The reaction mixture was then evaporated to dryness under reduced pressure. The crystalline residue was digested with 7 ml. of absolute ethanol, cooled, and the crystals removed by filtration to yield 2.68 Gm. (47%) of the product, m.p. 194–198°. After three recrystallizations from absolute ethanol, the hydrochloride melted at 200–201°; reported (17) m.p. 198–200°.

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Gravimetric Evaluation of Paper Chromatograms I

Theory, Instrumentation, and Procedure

By ALBERT E. H. HOUK

Theory, instrumentation, and procedure are given for the gravimetric determination of the distribution of compounds over an entire paper chromatogram. After the chromatogram is developed, the paper is cut into a series of $^3/_{16}$-in. strips at right angles to direction of development. The portion of the sample on each strip is eluted into a tared cup. The results are graphed, using the weights of the dried residues in the cups as the ordinates and the number of the strips from the bottom of the chromatogram as the abscissas. This procedure permits the quantitation and isolation of components in low concentrations which may be missed by conventional methods of detection and eliminates the need for a reference standard for quantitative evaluation. The isolated fractions may be used for additional studies.

PAPER CHROMATOGRAPHY has been used successfully for the separation and determination of chemicals in almost every field of science. Most of the standard procedures are inadequate since unknown impurities present may escape detection if they do not respond to the test used to detect the major components of the mixture. In many cases, the quantitative evaluation of the results is based on a visual comparison with reference standards (1–4).

In investigations in this laboratory of the purity of some U.S.P. reference standards and commercial drugs, these limitations of paper chromatography have been overcome by rapidly eluting fractions covering the entire paper chromatogram and by weighing the dried fractions obtained. The theory, instrumentation, and procedures developed and used during the past 3 years for obtaining weight-distribution curves from paper chromatograms are given. The application of the procedure to ouabain, other glycosides, and prednisone is presented in another paper (5).

THEORY

A solution of Sample A (Fig. 1) was streaked on the starting line, and the paper chromatogram developed by ascending technique in a sealed tank or by the continuous process (6). Where possible, volatile solvents were used as the mobile and immobile phases. Sample A was separated into four apparent components: *a*, *b*, *c*, and *d*. The chromatogram was cut into a series of narrow strips parallel to the starting line but were held together at the left edge by an uncut area. Components on the strips were eluted simultaneously into tared Teflon[1] cups. The solvent was evaporated, and the weight of the residue in each cup was determined. The results were graphed, using the weights of the residues as the ordinates and the number of the strip (counting from bottom of paper) as the abscissas. Usually, inflections obtained over those given by blank chromatograms indicated the presence of a component of the mixture, and its quantity was the sum of the weights from the strips producing the inflection less the weight given by the blanks or overlapping components.

The weight-distribution curve of Sample A shows that components *a*, *c*, and *d* were successfully isolated in a relatively pure form. The curve obtained for *b*, however, indicated the presence of more than one compound. The residues from the fractions in this area were combined, and a better separation of the components was achieved on another chromatogram with a modified solvent system.

INSTRUMENTATION

Sample Applicator Box (A, Fig. 2).—To prevent excessive evaporation of the more volatile immobile solvents from the chromatographic paper during application of sample to the starting line, a box of the following design is used: the box is 11 × 18 in. inside dimensions, and 5 in. in height at the sides and back; the front of the box is $4^1/_4$ in. in height, extending within $^3/_4$ in. to the top of the sides.

Place a crosspiece 4 in. from the inside front of the box and extending within $1^1/_2$ in. to the top. Inside and near the bottom of the front compartment thus formed, install two light sockets to take 25-watt light bulbs for back-lighting the starting line. Immediately above the light bulbs, insert a glass plate for heat insulation. Cut a groove around the inside periphery of the box $1^1/_2$ in. below the top and extending through the front panel to allow for the introduction of a glass plate to support the chromatographic paper. To prevent paper from adhering to the glass plate, it has been found advisable to cover the plate with a sheet of 100-gauge Teflon film[2] which can be replaced as needed. Place two $^5/_{16}$-in. glass rods (covered with closely fitting

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[1] Marketed by E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.

[2] Type A, FEP-fluorocarbon film, Film Department, E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.

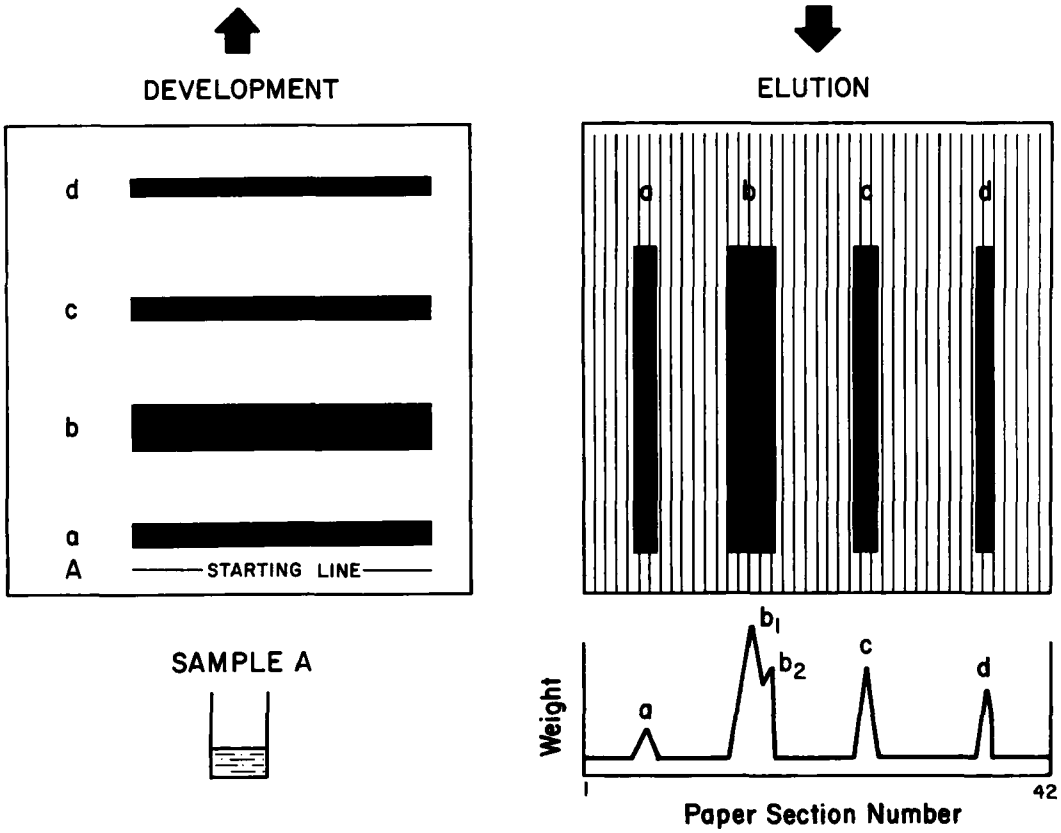

Fig. 1.—Determination of a weight-distribution curve of a paper chromatogram.

spaghetti tubing,[3]) 1/8 in. above the upper glass plate and 1 and 3 in., respectively, from the inside front of the box. Suspend these rods in the U-slots of two brackets made of 1/8-in. metal and fastened to the sides of the box. Cut grooves in the sides and back of the box 1/2 in. below the top to permit the introduction of a cover made from 1/16- in. aluminum sheet. Near the front of the cover, a slot 1/4 in. wide and 10 in. long is made so that (with the cover in position) the slot is midway between the glass rods. Fasten cross ribs to support blotting paper wetted with a suitable solvent to the underside of the cover. Mount the box on 3/4-in. thick blocks at each corner for ventilation.

Cutting Equipment (B, Fig. 2).—As a guide to be used in cutting the chromatogram, make a template 6 1/8 × 12 in. from 3/16-in. hardened aluminum plate. Using a milling machine equipped with a 1/32-in. circular saw, cut thirty 10-in. slots through the plate at 3/16-in. intervals, leaving a 1/2-in. border at the left and a 1-in. border at the top and bottom. With a scriber, make two guide lines at the top of the plate, perpendicular to and 3/8 and 1/2 in., respectively, below the top of the slits. Through the upper line, drill six 7/64-in. holes, approximately evenly spaced across the plate so that the top of the chromatogram can be brought even with the top line. Sometimes difficulties may be enountered in sawing the slots in the plate. In this case, several smaller sections (satisfactorily made) can be fastened together with metal strips at the top and bottom.

Place the paper and the template on a hardwood cutting board. Place two metal bars across the top and bottom of the template and bolt the extremities of the bars to the board to prevent the movement of the template during use. The bars should be long enough to permit the template to be moved across the entire width of the paper.

Weighing Cups (C, Fig. 2).—The weighing cups are made from disks of 50-gauge Teflon film 1 1/4 in. in diameter. Using a film 2 in. in width, attach the end of the film with adhesive tape to a cardboard strip 1 1/2 in. in width and make 50 superimposed layers of film around the strip. Fasten the end of the film with adhesive tape to the top layer on the cardboard. Place the cardboard strip with film between two sheets of bond paper and lay on a 1/4-in. sheet of polyethylene. Cut a block of disks using a 1 1/4-in. steel punch.

The molds for forming the cups are made from 13 1/2 × 2 7/8-in. strips of 1/2-in. hardened aluminum plate. Starting with the centers 2 1/4 in. from one end and 3/4 in. from either side of the strip, inscribe two parallel rows of nine 1 1/4-in. circles. At the center of each circle, drill a 15/32-in. hole through the plate. Remove sharp edges of the holes by reaming. For molding cups, make cylindrical plugs from 1/2-in. aluminum rod reduced in diameter on a lathe until it and a Teflon disk can be fitted easily but firmly into the holes of the mold. Cut plugs

[3] Teflon tubing, Pennsylvania Fluorocarbon Co., Inc. Philadelphia 4, Pa.

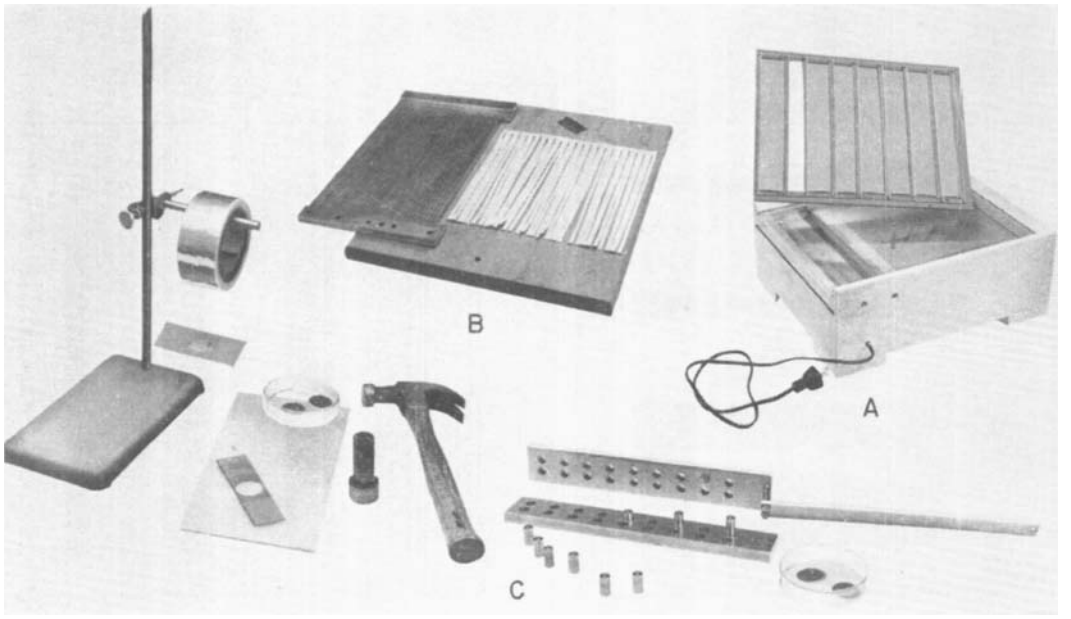

Fig. 2.—*A*, Sample applicator box; *B*, equipment for cutting paper chromatogram; and *C*, equipment for making Teflon weighing cups.

7/8 in. in length from the rod and round the edges with a cutting tool and a fine file. Polish both the holes of the mold and the plugs consecutively with 200-mesh aluminum oxide and talc wetted with alcohol.

To handle the hot molds, drill two 5/16-in. holes, 5/8 in. apart, 1/4 in. from the clear end of the mold. Make a detachable handle from a 14 × 1-in. strip of 1/4-in. aluminum. Attach to it two 1/4-in. brass rods so placed as to fit into the holes at the end of the mold.

Analytical Balance.—Balances with a suitable counterbalancing device and a sensitivity of 1 mcg. can be used for weighing the cups. The Cahn gram electrobalance[4] is satisfactory. However, an experimental automatic model 1700 AH electrobalance[4] (*A*, Fig. 3) developed for this study simplifies the weighing procedure greatly. This balance is connected to a precision four-digit voltmeter[5] which automatically indicated the weight. A 500-μc. radioactive ionizing unit is placed inside the weighing chamber to aid in the weighing of cups having a static charge. When weighing hygroscopic residues, two 5-ml. beakers containing P_2O_5 desiccant are placed in the weighing chamber.

Multifraction Elutor (B, C, Fig. 3).—The details of the construction of the component parts of the elutor canot be described adequately in a presentation of this type. Detailed drawings and instructions for making the complete elutor are available upon request to the author. The following is a functional description of this part of the equipment.

The assembled elutor is represented by *B*, Fig. 3. In *C*, Fig. 3, the elutor is pictured partly disassembled to show the component parts. The rigid frame work of the elutor consists of two sides and a bottom made of hardened aluminum plate. A crosspiece placed on either side lends rigidity to the sides Two bars are fastened to the bottom plate to prevent tipping.

A spacer bar (4, Fig. 3) made of extruded aluminum tee is used to hold the paper in position. The leg of the tee is sharpened to a wedge shape; the base of the tee is cut to have 21 slots on each side so positioned to accommodate the strips of paper. The spacer bar is placed on shelves fastened to the inside of the frame.

A cup holder (2, Fig. 3) containing four staggered rows of 12 holes each is placed on the bottom of the frame. The ends of the paper strips are held immediately above the proper cups by a needle rack (3, Fig. 3) fastened to the median line of the cup holder. To decrease the possibility of creeping of the eluting solvents, the cup holder and needle rack are coated with a TFE-fluorocarbon resin primer.[6] A flat heating element connected to the electric receptacle on the side of the frame is placed in the base of the frame under the cup holder to aid in the evaporation of the solvent collected in the cups.

A syringe rack (6, Fig. 3), carrying twenty-one 1-ml. syringes in two staggered rows, is mounted on each side of the frame. When in position, the tip of each syringe is in contact with a strip of paper. The plungers of the syringes are moved by crosspieces which are driven by a demountable clock motor and gears (8, Fig. 3).

To prevent the evaporation of volatile eluting solvents from the paper, two side panels (5, Fig. 3), equipped with metal ribs to hold sheets of blotting paper wetted with the solvent, are placed in grooves in the sides and base of the frame. A lid (7, Fig. 3) with a silicon rubber gasket is also provided so that with the side panels, the syringe racks, and the lid in place, the elutor is essentially airtight.

[4] Cahn Instrument Co., Paramount, Calif.
[5] Non-Linear Systems, Inc., Del Mar (San Diego), Calif.
[6] Finishes Division, E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.

PROCEDURE

Preparation of Cups.—Using disks prepared as described in the preceding section, center a disk in each of the circles on the mold. Form the cups by pushing the disks into the holes with the cylindrical plugs and leave the plugs in place. Using the mold handle, place the mold on its side in a muffle furnace at 365°. Avoid movement of plugs while the mold is hot. After 17 minutes, remove the mold, cool at room temperature for 5 minutes, then quench with cold water. Rinse the mold with 95% alcohol, remove plugs, and separate cups. Cups are usually made in lots of 1,000 and sorted by weight into groups covering a 1-mg. weight range.

The plug and plastic disk should fit snugly into the hole in the mold to prevent the plug from falling out and to assure bonding of the folds in the sides of the cup. If the plug is too large, it may stretch or tear the plastic disk. The heating time can be varied to achieve good bonding of the folds and to prevent the film from adhering to the mold. Detect improper bonding of the folds or the presence of small leaks by placing a few sample cups on a piece of absorbent paper and half filling each cup with methanol. Press the cups firmly against the absorbent paper with a smooth glass rod. Dry the rod and rotate it around the outside of the cups. If the rod adheres to the outside of the cup or a moist spot appears on the paper, a breakage of the film is indicated. Place a few crystals of a methanol-soluble fluorescent material in the cup and allow the solvent to evaporate overnight at room temperature. Appearance of capillary streaks up the sides of the cup or the presence of fluorescent material on the outside of the cup when it is examined under ultraviolet light indicates poor bonding or breakage of the film. Repetition of this test is unnecessary when satis-

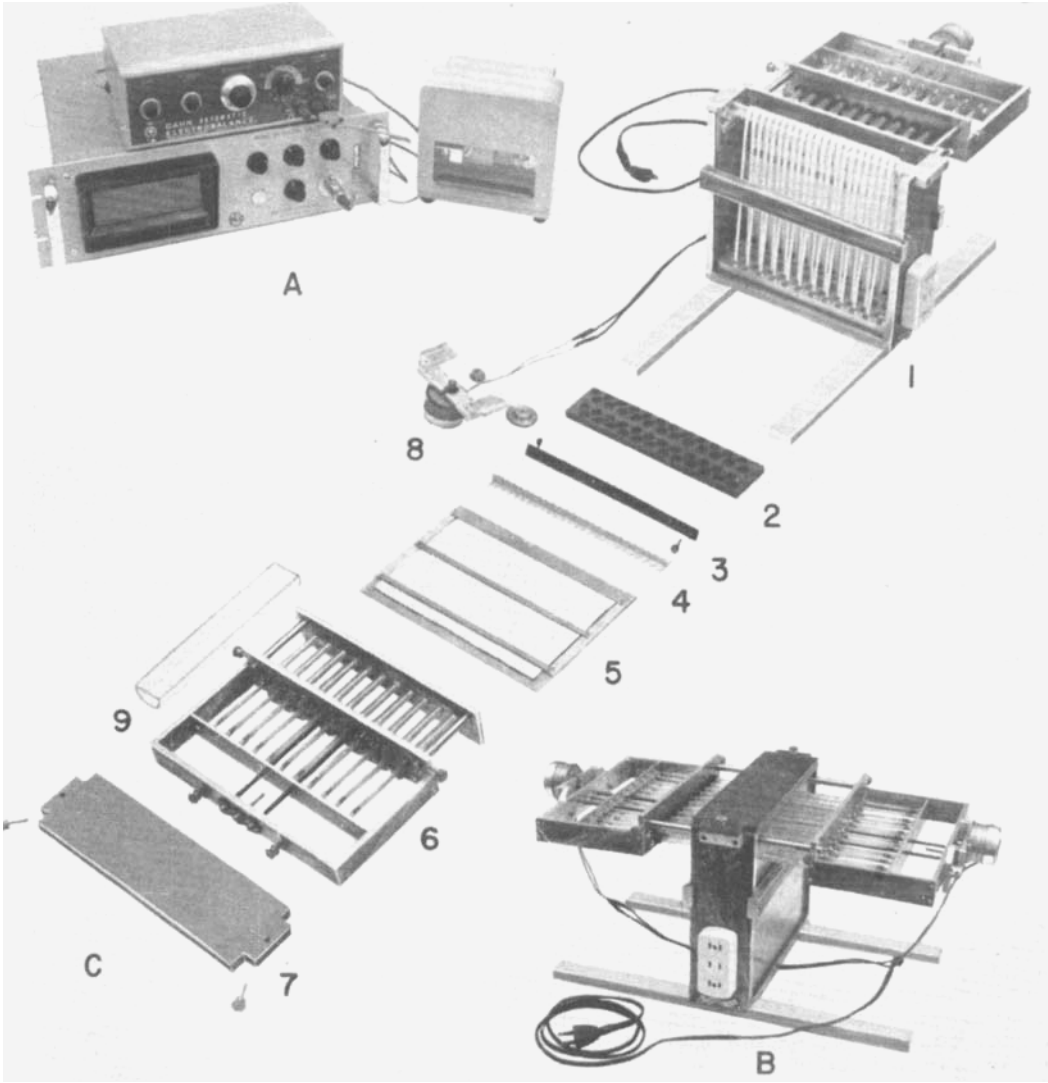

Fig. 3.—*A*, Automatic electrobalance with digital voltmeter; *B*, assembled; and *C*, breakdown view of multifraction elutor showing: 1, elutor with one side removed; 2, cup holder; 3, needle rack; 4, spacer bar; 5, side panel; 6, syringe rack; 7, lid; 8, motor with supporting frame; 9, glass trough.

factory conditions for producing suitable cups have been established.

Push the bottom of the cup outward by gentle pressure with a forceps so that the cups will rest securely on the balance loop. Immerse the cups successively in water, acetone, and petroleum ether; dry for 2 hours at 120° and store in a desiccator.

Preparation of Chromatographic Paper.—Draw lightly (with a lead pencil) a starting line 1 in. from the bottom of the paper, beginning $2^1/_2$ in. from the left edge and ending $^3/_4$ in. from the right edge of the paper.

Chromatographic paper may contain substances which will be removed by the eluting fluid and influence the weight of material determined. In most instances the major portion of these substances can be removed by successively washing the paper by descending chromatography with a solution of nitric acid in water (1–500), water, 95% ethanol, acetone, and petroleum ether. Dry the paper at room temperature and store to prevent contamination with dust or exposure to ammonia or other fumes. The procedure for washing the paper may be varied to suit the requirements of the problem.

Sometimes the successful separation of components of a mixture will require the addition of a chemical to act as an immobile phase. Since these chemicals may be removed from the paper by the eluting solvent, they should (whenever possible) be volatile or insoluble so that they will not contribute to the weight of the material in the cup after drying. When a nonvolatile substance is desirable as an immobile phase and it is soluble in the eluting solvent, correction for its weight may be possible through the use of a blank or from analysis of the residues in the cups. To prevent the contamination of the paper through handling following the application of the immobile phase, attach a glass rod to the top edge of the paper with Teflon-coated clips. This rod should be of the length required to support the paper in the development tank. After the paper has been saturated with the immobile phase, it is allowed to drain without blotting.

Application of Sample to the Paper.—Place the prepared paper in the applicator box (*A*, Fig. 2) with the starting line supported by the rods and immediately beneath the slit in the top of the box. If the paper carries a volatile material as the immobile phase, prevent excessive evaporation by wetting the blotting paper in the top of the box with the same material.

Weigh the desired quantity of the sample into a tared Teflon cup and dissolve in about 0.3 ml. of solvent. Using a weighed capillary tube with a fire-polished tip, transfer the sample solution to the paper by repeatedly streaking it uniformly along the entire length of the starting line. Rinse the cup and tube by adding two successive portions of about 0.1 ml. of the solvent and streak the resulting solutions on the starting line. The cup and the capillary tube are dried with the cups from the elution apparatus and reweighed to determine the weight of sample placed on the paper. Remove the paper from the box and evaporate the solvents used to dissolve the sample and the immobile phase by air drying in a hood briefly.

Development of Chromatogram.—The chromatogram may be developed by ascending chromatography using either a sealed tank or the continuous slit technique (6). If the latter technique is used, coating of the slit in the cover of the chamber with TFE-fluorocarbon resin primer[6] prevents the developing solvent from depositing some of the sample on the cover. Reattach the glass rod to the top of the paper after development. Remove the paper from the tank and evaporate excess mobile solvent by air drying in a hood. If the paper is to be cut after drying, wrap the paper loosely in Teflon-resin-coated aluminum foil and complete the drying in a vacuum oven. If the paper is to be cut while wet, place it between two Teflon sheets prior to cutting.

Cutting of the Paper.—Using the cutting equipment (*B*, Fig. 2), the paper is cut into strips at right angles to direction of development. Place the chromatogram and the template on the hardwood cutting board so that the right-hand edges coincide and the top edge of the paper is centered in the holes of the upper guideline of the template. Place the metal bars across the template to hold it in position. With either razor or surgical blades (the latter being preferred), cut paper into strips starting at the lower guideline. Any fractional strip remaining at the bottom of the chromatogram may be discarded.

Elution.—The required number of cups with a weight range of not more than 1 mg. are weighed to the nearest mcg. by counterbalancing and placed in the holes of the cup holder. Extra holes in the cup holder not required by the number of strips to be eluted can be used for controls which are desired. Attach the needle rack to the cup holder and place the holder in the elution apparatus.

With the aid of forceps, insert a $^1/_8$-in. aluminum rod immediately below the uncut portion of the chromatogram so that adjacent strips are on alternate sides of the rod. If Teflon sheets were used in cutting the chromatogram, they are now removed. With the aluminum rod, transfer the chromatogram to the spacer bar. When in position, adjacent strips should be on alternate sides of the spacer bar and in the appropriate slits. The tip of the strips should be about $^1/_8$ in. above the top of the cups. Adjust this distance by placing aluminum strips between each shelf and the ends of the spacer bar. Draw each strip taut and center it in front of the corresponding needle with metal forceps. Using a thin-walled glass tube of about $^1/_8$ in. in diameter, push the paper on the needle until the strip is centered over the cup.

The strips have a tendency to sag and buckle when wet with some eluting solvents. To prevent this, raise the spacer bar further by placing an additional metal strip $^1/_8$-in. thick between each end of the bar and the shelves. Thus the weight of the suspended cup holder exerts a constant tension on the strips.

Adjust the position of the movable crosspiece in each syringe rack so that the plungers can be pulled out slightly past the 1-ml. mark. Loosen the knurled knobs on the sides of the syringe rack, move the front plate back so that the ends of the syringes protrude about 1 in. from its face, and retighten the screws. Fill the trough (9, Fig. 3) with the eluting solvent. Immerse the ends of the syringes in the solvent. Remove the air in the syringes by filling and discharging the solvent. Finally, fill each syringe by drawing out the plunger until it is in

contact with the movable bar. Readjust the front plate of the carrier so that the syringes protrude about 1/2 in.

Moisten the blotter paper in the side panels with eluting solvent and put them in place. Insert the syringe racks into the appropriate slots and adjust so that the tips of the syringes are in firm contact with the centers of all strips. Attach the lid and the motor assemblies.

The motors are started after about 10 minutes to permit the partial saturation of the air in the elutor with the solvent. The time of elution can be varied by using different gears on the motor assembly. A short elution period is desirable with highly volatile, nonpolar solvents. The elution process can be automatically stopped when the plungers are near the bottom of the syringe barrel by powering the motors through a timer.

After allowing the solvent to drain from the strips into the cups for 10 minutes, remove the lid and the motor assemblies of the elutor. Adjust the syringe rack so the ends of the syringes will clear the spacer bar; remove the racks and the side panels. Evaporate the more volatile solvents by connecting the variable heater in the base of the elutor. Detach the needle rack from the cup holder and remove the spacer bar with attached paper and needle rack. Retain this assembly for additional studies which are indicated. Complete the drying of the cups by loosely wrapping the cup holder in aluminum foil and placing it in a vacuum oven. After drying, cool the cup holder to room temperature in a desiccating cabinet and reweigh the cups. Treat the results as indicated in the section on *Theory*.

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Gravimetric Evaluation of Paper Chromatograms II

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By ALBERT E. H. HOUK

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ANALYSES of steroid preparations by paper chromatography have been impeded by the difficulty in locating, rapidly eluting, and quantitating all components separated. This led to the development in this laboratory of new instrumentation and procedures reported previously (1). Results of some applications of this technique to steroid analyses are now given.

EXPERIMENTAL

Solvents

Neutral, readily volatile solvents were used. All solvents were percolated rapidly through a column of anion-cation exchange resin.[1] The first effluent through the column was discarded; the remainder was collected and redistilled. Formamide was redistilled under vacuum and stored in a desiccator over sulfuric acid until free of ammonia. Constant boiling fractions were returned to their original glass containers. Teflon[2] liners were placed in each bottle cap.

Chromatographic Paper

Whatman No. 3-mm. chromatographic paper in 8 × 8-in. sheets was ordinarily used. When required, 8 × 11 1/4-in. sheets were cut from a 23-cm. roll. Guidelines for streaking and development of paper were marked on all sheets; the paper was washed, dried, and stored (1).

Equipment

(*a*) Thomas-Mitchell chromatographic tank assembly with glass rods, troughs, and continuous technique accessories[3] was used with 8 × 8-in. sheets. The slotted cover and clips were coated with Teflon (1). (*b*) Thomas-Kolb chromatographic jar[3] was used with 8 × 11 1/4-in. sheets. A double-slotted cover, similar to the above accessory, was made for the jar and was coated with Teflon. (*c*) Two Pyrex 3-quart utility baking dishes,[4] each 9 1/4 × 14

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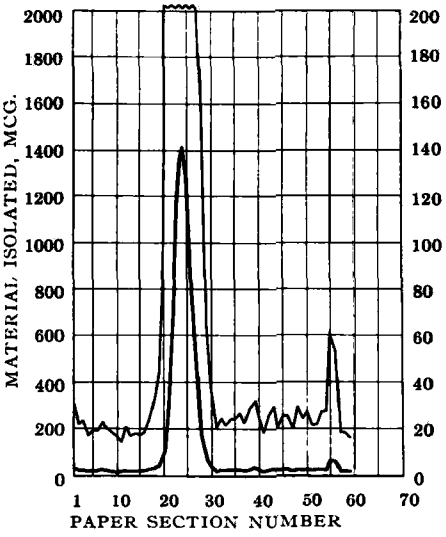

Fig. 1.—Weight-distribution curve of commercial ouabain (Sample A).

× 2½-in. and covered with a glass plate, were used to hold the immobile solvent during impregnation of the paper. (*d*) A drying rack similar to the commercial item[3] but 17 in. high; (*e*) desiccating cabinet[3] with glass trays and phosphorus pentoxide desiccant; (*f*) disposable plastic gloves[3] for handling the paper; (*g*) capillary melting point tubes,[5] each 1.2 mm. × 8 cm.-size and approximately 114 mg. weight. A 2-cm. section was broken off each and discarded; the remainder was fire polished. (*h*) Aluminum rod ⅛ × 10 in. was used to handle the cut chromatogram. (*i*) Thin-walled glass tube ⅛ × 6 in. was used to attach ends of paper strips to needle rack. (*j*) Sample applicator box, paper chromatogram cutting equipment, Teflon weighing cups, microbalance, and elutor apparatus (1).

General Procedure

Conclusions drawn from data obtained by this procedure are usually based on weights of small magnitude. Consequently, it is essential that precautions be taken to avoid nonvolatile contaminants. Glassware should be washed with chromic acid cleaning solution, thoroughly rinsed, drained, and stored in a dust-free cabinet. When in use, the equipment should be protected as far as possible from such contaminants as dust, fingerprints, chemical fumes, etc. The use of polyethylene gloves has been found helpful.

For initial quantitative analyses, three approximately equal aliquots of a sample were weighed in Teflon microcups. The first aliquot was used for gravimetric paper chromatography as previously described (1); the second aliquot was used to prepare a standard reference solution; the third aliquot was used as a control. To it and also to an empty, preweighed Teflon cup were added immobile, mobile, and eluting solvents equivalent approximately to that from an eluted paper strip. These cups were dried later with the cups from the elution apparatus and reweighed to determine nonvolatile components in solvents used, efficiency of drying, and weight changes of sample during processing. Chemical studies were conducted on solutions (equivalent to the reference solution) made from the residues in these cups and from the residues of the eluted fractions and the corresponding blank chromatogram. Thus both the recovery by weight and the recovery by chemical analysis of the fractions isolated were determined. Weighable residues remaining in the cup and capillary tube (after transfer of the sample to the paper for chromatography) were determined chemically also. Once a procedure for a sample was established, unnecessary control work was eliminated. Often the average of the blank areas of a sample chromatogram, eliminating the solvent front, was used to calculate the weight of the "fractions" above the "background weight."

Dry weight of the sample placed on the paper for fractionation normally agrees with summation of the dry weights of fractions isolated. If results indicate incomplete elution, the same chromatographic paper may be re-extracted, with the same or different solvent, into the same cups. Increases in weight of residue over increased blank confirm initial incomplete elution. There is no limit to the number of times the paper may be re-extracted, provided the solvent is evaporated between each operation. Further indication of the completeness of the extraction can be obtained by drying and dipping or spraying the eluted chromatogram with a developing reagent. Any fluorescence or color on the paper, characteristic of the sample and usually near the bottom of the strips, indicates incomplete elution.

The Teflon cups used to collect the fractions are chemically inert to most chemicals. Spot tests often were done directly in each cup. The cups were placed also in separate test tubes and color reactions completed there without transfer of residue.

Ouabain (Sample A).—Sample—7.556 mg. commercial ouabain (20.2% water) dissolved in water-methanol (1 + 2). Paper size—8 × 11¼ in. Immobile solvent—*n*-butanol-saturated water in meth-

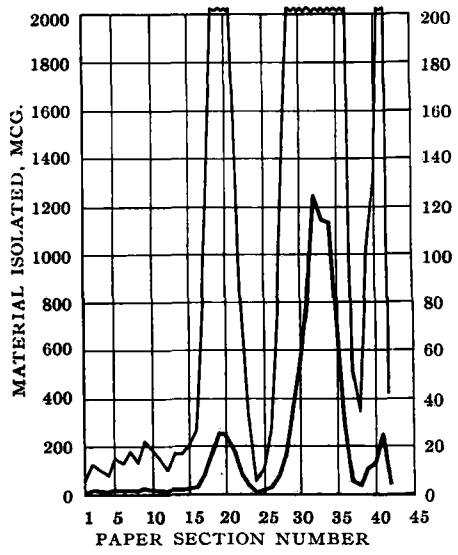

Fig. 2.—Weight-distribution curve of commercial ouabain (Sample B).

[5] Micro-Ware, Inc., Vineland, N. J.

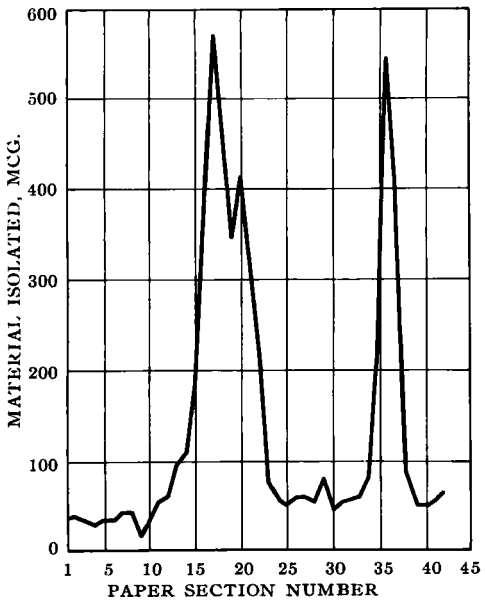

Fig. 3.—Weight-distribution curve of a mixture of ouabain, K-strophanthoside, and strophanthidin.

anol (3 + 1). Mobile solvent—water-saturated *n*-butanol. Development—20 hours at 28° by continuous ascending slit technique. Chromatogram—vacuum dried at 105° for 1 hour and cut into 59 strips. Elution—50% ethanol for 80 minutes. Drying—eluting solvent evaporated at 28° overnight (2), and residues vacuum dried in the presence of P_2O_5, at 130° for 5 hours.

Ouabain (Sample B).—Sample—9.999 mg. commercial ouabain (19.3% water) dissolved in water-methanol (1 + 2). Paper size—8 × 8 in. Immobile solvent—*n*-butanol-saturated water in acetone (1 + 1). Other techniques were as given under *Ouabain (Sample A)*, except the chromatogram was cut into 42 strips.

Mixture of Glycosides.—Sample—approximately 2 mg. each of ouabain (20.2% water), K-strophanthoside (strophosid; 4% water), and strophanthidin dissolved in methanol. Paper size—8 × 8 in. Immobile solvent—*n*-butanol-saturated water (paper was air dried until it appeared damp). Mobile solvent—water-saturated *n*-butanol. Development—to finish line by ascending technique in a sealed tank (approximately 4 hours at 28°). Chromatogram—vacuum dried at 105° for 1 hour and cut into 42 strips. Other techniques were as given under *Ouabain (Sample A)*.

Strophanthin K.—Sample—approximately 10 mg. commercial strophanthin K dissolved in water-methanol (1 + 2). Other techniques were as given under procedure for *Mixture of Glycosides*.

Digitoxin.—Sample—approximately 5 mg. commercial digitoxin dissolved in methanol-chloroform (1 + 3). Paper size—8 × 8 in. Immobile solvent—35% formamide in acetone. Mobile solvent—benzene-chloroform (1 + 1). Development—to finish line by ascending technique in a sealed tank (approximately 1 hour at 28°). Chromatogram—dried in a stream of air at 105° for 45 minutes and cut into 42 strips. Elution—50% aqueous pyridine for 60 minutes. Drying—solvent evaporated and residue dried in vacuum at 130° for 2 hours.

Prednisone.—Sample—approximately 3 mg. commercial prednisone dissolved in chloroform. Paper size—8 × 8 in. Immobile solvent—40% formamide in methanol. Mobile solvent—petroleum ether-chloroform (1 + 2). Development—2 hours at 28° by continuous ascending slit technique. Chromatogram—vacuum dried at 105° for 1 hour and cut into 42 strips. Elution—95% ethanol for 60 minutes. Drying—solvent evaporated and residue dried at 105° for 5 hours.

RESULTS

Figures 1 and 2 show the quantitative weight-distribution curves of two samples of ouabain. These are the purest and the most contaminated of eight samples examined for selecting reference standard material and evaluating the chemical assay procedures in current use. The left ordinate of each graph is for the lower curve, the right ordinate for the ten-fold expanded upper curve. The latter shows small weight variations in the base line.

On a dry weight basis, ouabain (Sample A) was recovered 100 ± 1% in the main fraction. Its "weight background" or base line averaged 22 mcg. per cup with extreme variations of ±8 mcg. The material recovered from the solvent front, where evaporation took place for 20 hours during development, weighed 81 mcg. A blank chromatogram gave an equivalent baseline and solvent front. On the basis of alkaline picrate color reaction (3), 99.5 ± 0.5% of the active component was recovered in the main fraction. The solvent front contained no chromogenic material. The extracted dried chromatogram sprayed with zinc chloride reagent (3) was negative.

With Sample B, 81.5 ± 1% of the starting material was recovered on a dry weight basis from the main fraction. When the alkaline picrate color reaction was applied to the unchromatographed material, the results indicated the presence of 87.4 ± 0.5% of ouabain. However, impurities less polar than ouabain and equal to 5.0% of the sample subjected to chromatography were found superimposed on the solvent front. This fraction gave a strong re-

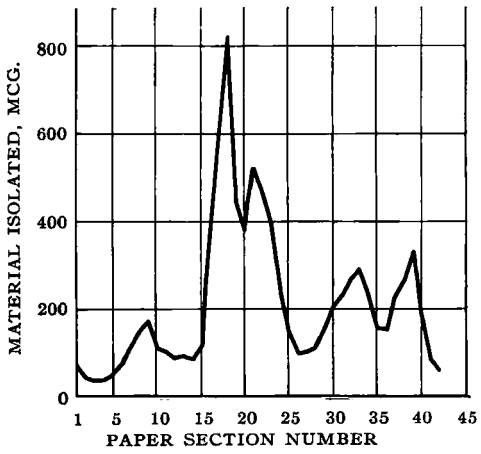

Fig. 4.—Weight-distribution curve of commercial strophanthin K.

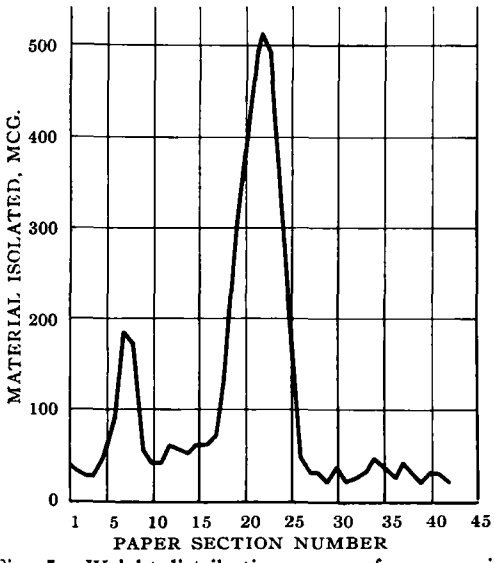

Fig. 5.—Weight-distribution curve of commercial digitoxin.

action with the alkaline picrate reagent and thus was included in the colorimetric assay of the unchromatographed sample. In addition to the above, impurities more polar than ouabain accounted for 13.4% of the dry weight of sample and gave little alkaline picrate color but had a strong anthrone color reactivity. We have occasionally observed the type of asymmetry seen at the apex of the main fraction. It usually disappears in subsequent weight-determination curves and may be associated with the physical characteristics of the zone and the finite width of the paper strips. The two examples cited above are typical of the quantitative recoveries which can be achieved by this technique.

Figures 3–6 show the type of general information that may be obtained from rapid qualitative weight-distribution measurements. Figure 3 shows the weight-distribution curve of a synthetic mixture of ouabain with two other glycosides, K-strophanthoside and strophanthidin. This figure illustrates the curve characteristics under two extreme conditions: (*a*) when a component is at or near the solvent front, and (*b*) when two components overlap. Strophanthidin (very close to the solvent front) gave a typical sharp inflection, indicating a buildup behind the solvent line. K-strophanthoside (slightly ahead of ouabain) easily showed its presence by a very sharp inflection above the ouabain background. A similar chromatogram when dried, sprayed with zinc chloride reagent, and viewed under ultraviolet light, showed only one fluorescent band for the ouabain-K-strophanthoside fraction. Where required, the fraction containing such an overlapping mixture could be rechromatographed, using a solvent system which would give a complete separation of the components.

Figure 4 shows a more complex type of weight-distribution curve obtained from strophanthin K. This sample was obtained as a possible pure reference standard. A chromatogram when dried, sprayed with zinc chloride reagent, and viewed under ultraviolet light, showed a series of overlapping fluorescent bands of varying intensity from the starting to the finishing line. It was difficult to estimate the quality of the product. However, the weight curve clearly showed it was unsuitable for a standard. It contained five major and five minor components; three were colored pigments with peaks near paper section numbers 8, 22, and 37. It was a mixture of glycosides similar to strophanthin N. F. X.

Figures 5 and 6 show the weight-distribution curves for commercial digitoxin and prednisone samples in which high boiling point formamide was used successfully as an immobile solvent. When the paper was dried before elution, it was difficult to remove completely the more nonpolar steroids because of adsorption by the paper. However, complete removal was readily obtained by cutting and eluting the wet paper with alcohol. The alcohol then was evaporated from the cups and the formamide removed under vacuum at 90°. The weight curve for digitoxin shows gitoxin near the starting line and traces of two components near paper sections numbers 12 and 15 more polar than digitoxin. The two minor components had not been observed previously by conventional paper chromatography but were confirmed by high concentration chromatography and use of slit technique. The weight curve for prednisone indicates absence of marked impurities. It does show a broad solvent front due to evaporation of the highly volatile mobile solvents within the developing chamber and below the slit of its lid. Multiple solvent fronts, due to different evaporation points from the paper by mixed mobile solvents, were no problem in these systems.

DISCUSSION

In conventional chromatography, it is becoming more important to control the purity of the paper and solvents used. It is critical in the gravimetric procedure herein described, since the presence of nonvolatile, soluble contaminants influences the accuracy of quantitative studies and may obscure the presence of minor components of the sample. Washing with acetone removed 2.8 mg. of soluble material from one sheet of Whatman No. 1 filter

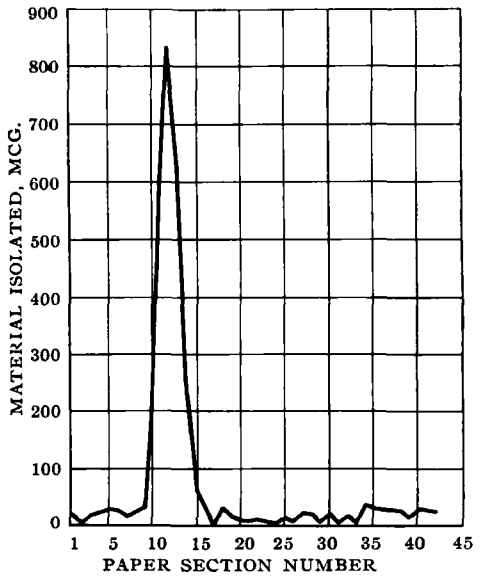

Fig. 6.—Weight-distribution curve of prednisone.

paper.[6] Total and acetone-soluble materials were even higher in Whatman No. 3 paper. These substances are particularly troublesome when aqueous solvents are used as eluents.

Several precautions may aid in controlling this difficulty. The amount of extraneous material eluted varies with the volume of solvent used. Therefore, the eluting solvent should be kept to the *minimum* required for the complete removal of the components of the sample. The selection of solvent systems which tend to concentrate the fraction to be studied in a narrow band reduces the amount of background material since a smaller area of paper is eluted to obtain the desired fraction.

When it is sufficient to measure the sum of the minor components, the accuracy can be enhanced by using the slit technique and concentrating the less polar materials at the solvent front. Similarly, the more polar components may be concentrated on or near the starting line by the proper choice of solvents.

The limit of sensitivity of the method may be determined by comparing the weight of the sample used and the weight of the background. For example, in ouabain (Sample A), a 10-mg. sample was used and the background weight determined on a blank chromatogram averaged 22 mcg. per cup with extreme variations of ±8 mcg. Since these extremes differ by a maximum of 16 mcg., impurities present in quantities of more than 16 mcg. or 0.16% of a 10-mg. sample and concentrated on a single strip, should produce discernible peaks. The sensitivity is reduced where the impurity is distributed over two or more strips.

If one is interested only in the amount of the main component present, it is not necessary to elute quantitatively the entire chromatogram. The desired result can be obtained by comparing the dry weight of the sample and the dry weight of chief component eluted from the paper.

The elution apparatus described was designed to accommodate an 8 × 8-in. paper cut into 42 strips. Obviously, shorter chromatograms can be used equally well. Long paper may also be used by cutting it into two or more sections and eluting each section separately. In using small samples it may be desirable to use thinner or more narrow paper and with large samples to increase the weight or width of the paper. In the latter case, it will be necessary to increase the height of the elution equipment and the capacity of the cups.

[6] Private communication from L. C. Mitchell of the Food and Drug Administration.

SUMMARY

In a previous publication (1), theory, instrumentation, and procedure were given for the gravimetric determination of the distribution of compounds over an entire paper chromatogram. Some applications of this technique to analysis of steroids are now reported. The method measures components of a drug or other product which are separated by paper chromatography and may be missed by conventional techniques of detection. It eliminates the need for reference standards which for impurities or decomposition products may not be readily available. Precautions in using the method are given. Upon completion of the gravimetric analyses, the isolated fractions were used for additional studies.

REFERENCES

(1) Houk, A. E. H., THIS JOURNAL, 52, 738(1963).
(2) Trivedi, J., Shell, J. W., and Biles, J. A., *ibid.*, 48, 583(1959).
(3) "United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, p. 482.

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ALTHOUGH NUMEROUS investigators have studied the effects of narcotics on metabolic processes of the brain *in vitro*, the mechanisms by which these drugs evoke pharmacologic effects are not well understood. It has been demonstrated that barbiturates inhibit oxidation of carbohydrate by brain slices and minces (1–4). Other studies have revealed indications that these drugs interfere with hydrogen transport (5, 6) and with the oxidation of pyruvate (7). It has furthermore been shown that barbiturates in concentrations which more closely approach those present in the brain during anesthesia uncouple oxidation from phosphorylation *in vitro* (8).

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TABLE I.—EFFECT OF CERTAIN HYPNOTICS ON RESPIRATION OF HOMOGENATES[a]

Substrate	Drug	Concn.	μl. O_2 Uptake/ 90 min.[b]	Increase (+) or Decrease (−) Due to Drug, %
None	None	...	20 ± 2.2	...
None	Pentobarbital	1×10^{-3} *M*	12 ± 1.4	−40
None	Pentobarbital	5×10^{-4} *M*	16 ± 0.9	...
None	Glutethimide	1×10^{-3} *M*	14 ± 0.6	−30
None	Glutethimide	5×10^{-4} *M*	16 ± 1.2	...
None	Methyprylon	1×10^{-3} *M*	21 ± 1.9	...
None	Ethinamate	1×10^{-3} *M*	19 ± 2.1	...
Glucose	None	...	145 ± 7.2	...
Glucose	Pentobarbital	1×10^{-3} *M*	40 ± 2.5	−72
Glucose	Pentobarbital	5×10^{-4} *M*	77 ± 3.8	−47
Glucose	Glutethimide	1×10^{-3} *M*	85 ± 5.3	−41
Glucose	Glutethimide	5×10^{-4} *M*	118 ± 4.2	−19
Glucose	Methyprylon	1×10^{-3} *M*	142 ± 5.8	...
Glucose	Ethinamate	1×10^{-3} *M*	141 ± 5.3	...
Pyruvate	None	...	79 ± 4.2	...
Pyruvate	Pentobarbital	1×10^{-3} *M*	31 ± 2.0	−60
Pyruvate	Pentobarbital	5×10^{-4} *M*	41 ± 1.4	−48
Pyruvate	Glutethimide	1×10^{-3} *M*	51 ± 3.8	−35
Pyruvate	Glutethimide	5×10^{-4} *M*	73 ± 4.2	...
Pyruvate	Methyprylon	1×10^{-3} *M*	77 ± 4.0	...
Pyruvate	Ethinamate	1×10^{-3} *M*	77 ± 3.3	...
Malate	None	...	30 ± 2.9	...
Malate	Pentobarbital	1×10^{-3} *M*	21 ± 1.6	−30
Malate	Pentobarbital	5×10^{-4} *M*	23 ± 1.0	−23
Malate	Glutethimide	1×10^{-3} *M*	22 ± 2.4	−26
Malate	Glutethimide	5×10^{-4} *M*	23 ± 2.5	−23
Malate	Methyprylon	1×10^{-3} *M*	29 ± 1.6	...
Malate	Ethinamate	1×10^{-3} *M*	28 ± 1.1	...
Glutamate	None	...	33 ± 3.0	...
Glutamate	Pentobarbital	1×10^{-3} *M*	23 ± 1.9	−30
Glutamate	Pentobarbital	5×10^{-4} *M*	28 ± 1.4	...
Glutamate	Glutethimide	1×10^{-3} *M*	26 ± 2.1	−21
Glutamate	Glutethimide	5×10^{-4} *M*	30 ± 2.1	...
Glutamate	Methyprylon	1×10^{-3} *M*	31 ± 1.4	...
Glutamate	Ethinamate	1×10^{-3} *M*	31 ± 2.2	...

[a] Experimental conditions as outlined in the text. [b] Standard deviation. Results significant at the 95% probability level as shown by the Student "t" test.

More recently the hypnotic drugs glutethimide,[1] methyprylon,[2] and ethinamate[3] have been introduced. A search of the literature indicates that the effects of these drugs on metabolic processes of the brain have not been extensively studied. Accordingly, a series of experiments was designed to ascertain whether these drugs evoke effects similar to those of pentobarbital on brain homogenates and mitochondrial preparations.

EXPERIMENTAL

Young, adult Sprague-Dawley rats of both sexes were used as the experimental animals. Each rat was stunned and decapitated, the brain was immediately removed, washed in cold 0.1 *M* phosphate buffer, blotted dry, and weighed on a torsion balance. The brain was then ground in a Ten Broek homogenizer containing sufficient cold 0.1 *M* potassium phosphate buffer, pH 7.4, so that there were 100 mg. of brain per ml. of homogenate.

Mitochondria were isolated from homogenates of whole brain prepared in cold 0.25 *M* sucrose solution according to the method of Brody and Bain (8). Centrifugation was accomplished in a Servall RC-2 centrifuge equipped with an SS-34 rotor. The mitochondrial suspension was adjusted with 0.25 *M* sucrose solution so that each milliliter contained mitochondria representing 200 mg. of brain, wet weight.

Conventional manometric techniques were used for measuring oxygen consumption. In the preparation of the homogenates no more than 10 minutes elapsed between the time the animals were sacrificed and the flasks placed in the bath. All results represent the average of determinations obtained with eight rats and duplicate flasks were used in each instance. The pH of the contents of a representative number of flasks was determined at the end of the experiments to insure that the observed effects were not due to a change in pH.

All flasks used in studying respiration of the homogenates contained in the main compartments 1.0 ml. of 0.1 *M* potassium phosphate buffer (pH 7.4), 0.3 ml. 5×10^{-4} *M* cytochrome c, 0.5 ml. of homogenate, and 0.3 ml. of drug solution to yield the concentrations listed in Table I. Four-tenths of a milliliter of 0.1 *M* substrate was tipped from the sidearm at zero time and the center wells contained 0.2 ml. of 10% KOH together with a strip of filter paper.

When endogenous uptake was determined, water rather than substrate was added from the sidearm.

[1] Marketed as Doriden by Ciba Pharmaceutical Products, Inc.
[2] Marketed as Noludar by Hoffmann LaRoche, Inc.
[3] Marketed as Valmid by Eli Lilly and Co.

TABLE II.—EFFECT OF CERTAIN HYPNOTICS ON OXIDATIVE PHOSPHORYLATION BY RAT BRAIN MITOCHONDRIAL PREPARATIONS WITH PYRUVATE AS SUBSTRATE[a]

	Concn. Drug	μA Oxygen Consumed[b]	μM Phosphate Uptake	P/O Ratio
Controls		1.6 ± 0.3	4.3 ± 0.7	2.7 ± 0.2
Glutethimide	1×10^{-3} M	0.8 ± 0.1	2.1 ± 0.2	2.6 ± 0.3
Methyprylon	1×10^{-3} M	1.6 ± 0.3	4.2 ± 0.3	2.6 ± 0.2
Ethinamate	1×10^{-3} M	1.6 ± 0.2	4.1 ± 0.4	2.5 ± 0.2
Pentobarbital	5×10^{-4} M	1.1 ± 0.2	0.8 ± 0.1	0.7 ± 0.1

[a] Experimental conditions described in text. Each result represents the average obtained with six different animals. [b] Standard deviation; results significant at the 99% probability level as shown by Student "t" test.

With glucose as the substrate the flasks contained the reagents listed above plus 0.1 ml. of 5×10^{-2} M adenosine triphosphate (ATP) and 0.1 ml. of 10^{-2} M $MgCl_2$ in the main compartment, and 0.1 ml. of 10^{-3} M DPN added with the substrate from the sidearm. In the experiments with pyruvate as the substrate flasks contained, in addition to the above reagents, 0.1 ml. of 5×10^{-2} M ATP; 0.1 ml. of 10^{-2} M $MgCl_2$ and 0.1 ml. of 2.5×10^{-2} M nicotinamide all added to the main compartment, while the pyruvate plus 0.1 ml. of 10^{-2} M malate was added from the sidearm.

Water was used to make the final volume of flask contents 3.0 ml. and, with the exception of the experiments with malate which were run at 30°, the temperature of the bath was 37°. The equilibration period was 10 minutes after which the manometers were closed and readings taken at 15-minute intervals for 90 minutes.

Flasks used in the study of oxidative phosphorylation contained, in the main compartment, 0.4 ml. of mitochondrial suspension; 0.5 ml. of 0.1 M phosphate buffer, pH 7.4; 0.1 ml. of 5×10^{-4} M cytochrome c; 0.4 ml. of 0.1 M glycylglycine buffer, pH 7.4; 0.1 ml. of 1.5×10^{-2} M DPN; 0.1 ml. of 0.5 M KCl; 0.1 ml. of 0.3 M $MgCl_2$; 0.1 ml. of 0.8 M glucose; 0.1 ml. of hexokinase solution containing 600 units per ml.; 0.1 ml. of 5×10^{-2} M ATP; and 0.1 ml. of 0.5 M NaF. Three-tenths of a milliliter of 0.1 M pyruvate together with 0.1 ml. of 10^{-2} M malate was added as substrate. Three-tenths of a milliliter of drug solution was added to yield the concentrations listed in Table II. The center wells contained 0.2 ml. of 10% KOH together with a strip of filter paper, and the temperature of the bath was 30°.

The control flasks, which contained water in place of drug solution, were removed at the end of the 10-minute equilibration period and immediately placed in ice, following which 0.5 ml. of chilled 20% trichloracetic acid (TCA) was added to denature proteins. The same procedure was followed with the flasks containing the drugs after the oxygen uptake had been measured for 30 minutes. The contents of the TCA-treated flasks were pipetted into centrifuge tubes and the denatured protein separated by centrifugation at 3000 × g for 10 minutes. Inorganic phosphate was determined in the supernatant fluid by the method of Fiske and SubbaRow (9). A Beckman model DU spectrophotometer was used and the absorbance was determined at 660 mμ. The inorganic phosphate uptake was determined as the difference between the amount of phosphate in the vessels to which TCA had been added at the end of the equilibration period and those to which TCA was added after measuring oxygen uptake for 30 minutes. The phosphate-to-oxygen ratio was calculated as the ratio of micromoles of inorganic phosphate uptake to the microatoms of oxygen consumed, in conformance to the methods followed by Brody and Bain (8).

The effect of the drugs on malic dehydrogenase activity was determined by the method of Ochoa (10). The oxidation of dihydro-β-diphosphopyridine nucleotide (DPNH) to diphosphopyridine nucleotide (DPN) with the concomitant reduction of oxaloacetate to malate was measured, in the presence and in the absence of the drugs, by following the decrease in absorbance at 340 mμ with the Beckman model DU spectrophotometer. In these experiments the reaction mixture contained, in a total volume of 3.0 ml., 40 units of malic dehydrogenase, 5×10^{-5} M DPNH, 5×10^{-5} M oxaloacetate, and 10^{-3} M of the various drugs.

In those instances where the drugs produced an inhibitory effect on the respiration of homogenates, additional experiments were performed to determine

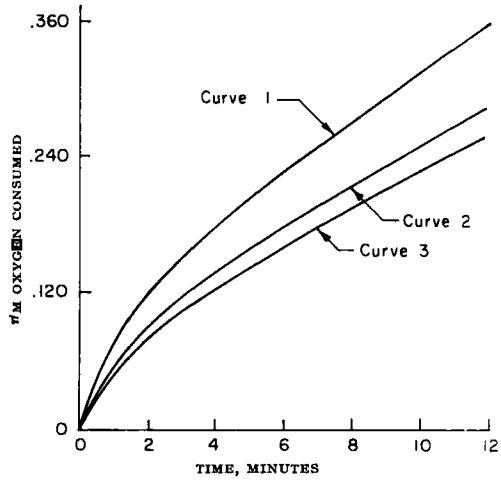

Fig. 1.—The effect of pentobarbital and glutethimide on respiration of brain mitochondria in the presence of malate. Cell of the polarograph contained 0.5 ml. of 0.01 M phosphate buffer; 0.1 ml. of 5×10^{-4} M cytochrome c; 0.1 ml. of 0.05 M ATP; 0.1 ml. of 0.5 M KCl; 0.1 ml. of 1.5×10^{-2} M DPN; 0.1 ml. of 10^{-2} M $MgCl_2$; 0.3 ml. of 0.1 M malate; and 0.2 ml. mitochondrial suspension representing 100 mg. brain. Drug, when present, added as 0.2 ml. of 10^{-2} M. Water added to make volume 2.0 ml. Temperature 25°. Results represent averages obtained with six animals. Key: curve 1, control (no drug added); curve 2, glutethimide; and curve 3, pentobarbital.

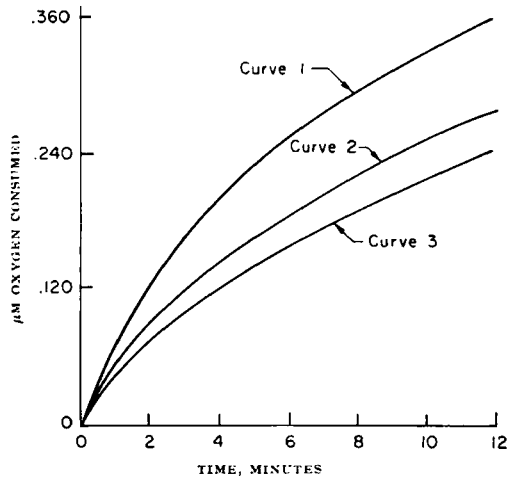

Fig. 2.—The effect of pentobarbital and glutethimide on respiration of brain mitochondria in the presence of pyruvate. Cell contents same as outlined in Fig. 1 with 0.3 ml. of 0.1 *M* pyruvate added. Experimental conditions identical to those used for Fig. 1. Results represent averages obtained with six animals. Key: curve 1, control (no drug added); curve 2, glutethimide; and curve 3, pentobarbital.

the effects of the rate of oxygen uptake by mitochondria. This was carried out by measuring the oxygen uptake polarographically, using a vibrating platinum electrode instrument[4] adapted from the method outlined by Chance (11). With such an instrument it was possible to measure the consumption of extremely small amounts of oxygen accurately over very short time intervals. The effects of pentobarbital and glutethimide on the respiration of mitochondria in the presence of malate, pyruvate, or DPNH as substrates were thus determined.

RESULTS

The data included in Table I indicate that either pentobarbital or glutethimide, at concentrations of 10^{-3} *M* and 5×10^{-4} *M*, inhibit the endogenous oxygen uptake of homogenates as well as oxygen uptake in the presence of added glucose, pyruvate, malate, or glutamate. Neither ethinamate nor methyprylon (at these concentrations) evoked appreciable effect. As further shown by Table I, pentobarbital produced somewhat more marked effects on respiration than did glutethimide.

Figures 1–3 illustrate the effects of pentobarbital and glutethimide on the oxidation of malate, pyruvate, and DPNH by brain mitochondrial preparations. It is apparent that both glutethimide and pentobarbital significantly depress the rate of oxidation of these substrates.

Table II shows that, of the four drugs studied, only pentobarbital evoked significant effects on oxidative phosphorylation by brain mitochondria.

None of the drugs produced significant effects on the activity of a purified malic dehydrogenase obtained from commercial sources.[5]

[4] Model K Oxygraph, Gilson Medical Electronics Co., Madison, Wis.
[5] Sigma Chemical Co., St. Louis, Mo.

DISCUSSION

The oxidation of malate and glutamate by homogenates was inhibited by pentobarbital or glutethimide. Other investigators (7) have shown that pentobarbital does not block the oxidation of glucose to pyruvate. It has also been demonstrated that the major pathway for the metabolism of glucose by the brain occurs *via* glycolysis to pyruvate (12) with subsequent oxidation by way of the tricarboxylic acid cycle. It might be expected, therefore, that the inhibitory effect evoked on the oxidation of glucose would be very similar in magnitude to the effect on the oxidation of pyruvate. In this study it was found that pentobarbital inhibited the oxidation of glucose to about the same degree as was observed with pyruvate.

Although the oxidation of the substrates included in this study is dependent upon specific dehydrogenase systems, it is not correct to deduce that the inhibitory effects produced by the drugs are due to interference at the dehydrogenase level because the observations made with homogenates are not necessarily the same as those made with purified enzyme systems. The use of more discrete preparations, such as mitochondria, affords a means of measuring oxidation free from the influence of many of the reactions which simultaneously occur in homogenates. Polarographic determination of oxygen consumption by mitochondria showed that pentobarbital and glutethimide were inhibitory in the presence of added malate, pyruvate, or DPNH, while the activity of a purified malic dehydrogenase preparation was not altered. Such observations

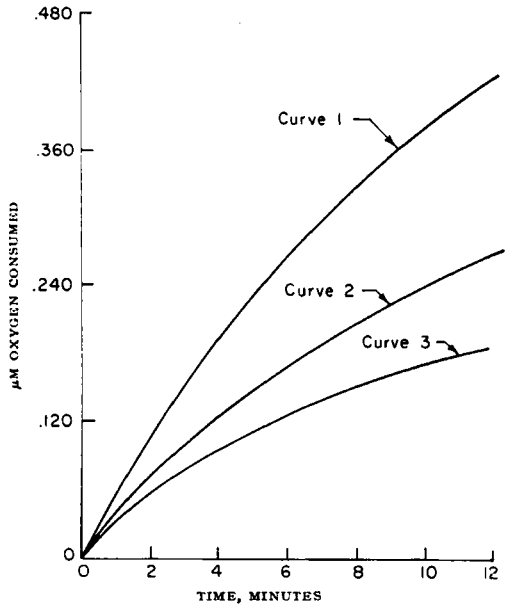

Fig. 3.—The effect of pentobarbital and glutethimide on the oxidation of reduced diphosphopyridine nucleotide (DPNH). Cell contents same as those for Fig. 1 except that the only substrate added was 0.3 ml. of 10^{-2} *M* DPNH. Experimental conditions identical to those used for Fig. 1. Results represent averages obtained with six animals. Key: curve 1, control (no drug added); curve 2, glutethimide; and curve 3, pentobarbital.

indicate that dehydrogenases are not directly inhibited by these drugs and lead to a consideration of possible effects of the drugs at the level of hydrogen transport.

It has been well established that mitochondria are rich in the enzymes of the tricarboxylic acid cycle, the electron transport and accompanying phosphorylating systems, and the cofactors associated with these enzymes (13, 14). The malate and pyruvic dehydrogenases both require DPN as a cofactor and utilize the same cytochrome c reductase system as the primary pathway for electron transport. Thus, the main difference between the oxidation of pyruvate and malate involves the dehydrogenases which transfer protons and electrons from these substrates to DPN. Pentobarbital or glutethimide, then, may conceivably produce an inhibition of oxygen uptake in the presence of malate or pyruvate by blocking at the dehydrogenase level or at some point in the hydrogen transport system beyond the step responsible for the reduction of DPN. The data in Fig. 3 reveal that the rate of oxygen uptake in the presence of added DPNH was inhibited by both pentobarbital and glutethimide.

The succinic oxidase system, in the presence of cytochrome c, will oxidize *p*-phenylene diamine with electrons being transferred directly to the cytochrome c, thus involving a bypass of the succinate-cytochrome b complex (15). It has been demonstrated that glutethimide (16) or pentobarbital (17) do not inhibit the oxidation of succinate or of *p*-phenylene diamine. It may be inferred, therefore, that the dehydrogenases which act on *p*-phenylene diamine or succinate transfer electrons to a point beyond the DPNH-dehydrogenase step in the electron transport system. There is evidence (18) that pentobarbital does not affect the DPN-linked dehydrogenases of the tricarboxylic acid cycle. The results of this study indicate that neither pentobarbital nor glutethimide inhibit the activity of a purified malic dehydrogenase system. However, the drugs inhibit the oxidation of DPNH and of other substrates which require DPN-linked dehydrogenases. Therefore, the results reported herein suggest that the effects produced by either pentobarbital or glutethimide *in vitro* are elicited at the level of the electron transport system between DPN and cytochrome c.

Of the four drugs studied, only pentobarbital was found to uncouple oxidation from phosphorylation. The concept of such uncoupling seems to be more widely accepted as a possible explanation of central nervous system depression than is the inhibitory effect of this drug on respiratory enzymes, perhaps because concentrations required to impair oxidative phosphorylation *in vitro* more closely approach the concentrations which produce *in vivo* effects. It is pertinent to point out, however, that not all depressants of the central nervous system are uncoupling agents, as shown by the fact that neither chloral hydrate nor paraldehyde uncouple oxidation from phosphorylation (19).

It seems of interest to note that neither ethinamate nor methyprylon had an effect on the oxidative processes included in this study even though the hypnotic potency of these drugs has been reported to be comparable to that of glutethimide (20–22).

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Some Observations on the Kinetics of the C·4 Epimerization of Tetracycline

By EDWARD G. REMMERS, GEORGE M. SIEGER, and ALBERT P. DOERSCHUK

The C·4 epimerization of tetracycline has been shown to follow the kinetics of a first-order reversible reaction. Phosphate and citrate ions increased the rate of C·4 epimerization. The reaction rate in either 1.00 molar phosphate or 1.00 molar citrate buffer at pH 4.0 and 23° was approximately 70 times the rate observed in distilled water under the same conditions of temperature and pH. An Arrhenius plot indicated that the apparent energy of activation for the C·4 epimerization of tetracycline at pH 4.0 and 0.10 molar phosphate buffer was approximately 20 kilocalories/gram-mole. The equilibrium concentration and the rate at which equilibrium was approached were dependent on the pH. Of the five pH levels studied, a maximum C·4 epimer level of 55 per cent at equilibrium occurred at pH 3.2. Of the five pH levels studied, the rate at which equilibrium was approached was highest at pH 3.2, 4.0, and 5.0.

In 1955 Doerschuk, *et al.* (1), observed a reversible isomerization in members of the tetracycline family. This observation was confirmed by Stephens, *et al.* (2), in 1956 and by Kaplan, *et al.* (3), in 1957. The initial observations reported by Doerschuk, *et al.* (1), were extended by McCormick, *et al.* (4, 5), in a series of papers in which the preparation, properties, and proof of structure of several 4-epi-tetracyclines were described. In the paper by McCormick, *et al.* (5), a number of experimental conditions permitting the epimerization of members of the tetracycline family was presented with values of the equilibrium concentration of C·4 epimer in a variety of solvent systems.

The purpose of this paper is to describe the kinetics of the C·4 epimerization in solutions displaying pharmaceutical utility. A study of this type provides useful background information in formulating stable solutions of tetracycline.

EXPERIMENTAL

Analytical Procedure.—The analytical procedure used in this study for measuring 4-epi-tetracycline was a slight modification of the absorbancy-ratio assay described by McCormick, *et al.* (5). The assay is based on the observation that the ratio of absorbancies at 254 and 267 mμ differs for tetracycline and its C·4 epimer. To measure small amounts of C·4 epimer precisely, the original absorbancy ratio assay was modified by F. S. Chiccarelli (private communication) so that it might be run as a differential assay. Samples of the standards for tetracycline·HCl and 4-epi-tetracycline (ammonium salt) were obtained from F. S. Chiccarelli, Assay Development Department.

Experimental Procedure.—Solutions of various buffers were prepared and adjusted to the desired pH. The buffers were allowed to equilibrate at the desired temperature in a volumetric flask before tetracycline·HCl or its C·4 epimer was dissolved in the buffer. At frequent intervals aliquots were withdrawn to determine the C·4 epimer content by the method described above.

Estimation of Rate Constants.—The reaction rate constants for the C·4 epimerization were estimated from the equation describing the kinetics of a first-order reversible reaction

$$ln \frac{Ao - Ae}{A - Ae} = (k_1 + k_{-1})\, t \qquad \text{(Eq. 1)}$$

where Ao = per cent tetracycline·HCl at $t=o$; A = per cent tetracycline·HCL at $t=t$; Ae = per cent tetracycline·HCl at equilibrium; t = time, hours; k_1 = forward reaction rate constant, hours^{-1}; and k_{-1} = backward reaction rate constant, hours^{-1}.

Throughout this paper the symbol k_1 is used for the reaction rate constant for the forward reaction of tetracycline epimerizing to 4-epi-tetracycline. The symbol k_{-1} is used for the backward reaction of 4-epi-tetracycline to tetracycline. The symbols for the two reaction rates are used for the directions of the reactions as indicated above even though the principal starting material in the reaction may be 4-epi-tetracycline in some experiments.

Equation 1 indicates that if the logarithm of the reciprocal of the fraction of equilibrium attained at time t is plotted as a function of time, a straight line should be obtained whose slope is equal to $(k_1 + k_{-1})$. The method of least squares was used to estimate the slope of the straight line passing through the experimental points, thereby providing an estimate of $(k_1 + k_{-1})$. The individual rate constants k_1 and k_{-1} are readily determined from the equilibrium concentration of tetracycline using the relationship

$$\frac{1 - Ae}{Ae} = \frac{k_1}{k_{-1}}$$

The 95% confidence limits for k_1 and k_{-1} were determined from the least squares calculations presented in standard statistical texts. For all other computations of slopes and intercepts the method of least squares was used.

RESULTS AND DISCUSSION

Determination of Reaction Order for the Epimerization of Tetracycline to 4-epi-Tetracycline.—

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TABLE I.—EXPERIMENTAL DISAPPEARANCE RATES AND AVERAGE CONCENTRATIONS FOR TETRACYCLINE DURING FIRST 8.5 HOURS OF EPIMERIZATION

Initial Concn. of Tetracycline and C·4 Epimer	Tetracycline, Initial %	Tetracycline after 8.5 Hr., %	Rate of Disappearance $-\frac{\Delta(\text{Tetracycline})}{\Delta t}$	Average Concn. of Tetracycline During First 8.5 Hr. of Reaction
250 mcg./ml.	96.80	92.78	1.182 mcg./ml.-hr.	237.0 mcg./ml.
500 mcg./ml.	96.80	92.20	2.706 mcg./ml.-hr.	472.5 mcg./ml.
1,000 mcg./ml.	96.80	91.95	5.706 mcg./ml.-hr.	943.8 mcg./ml.

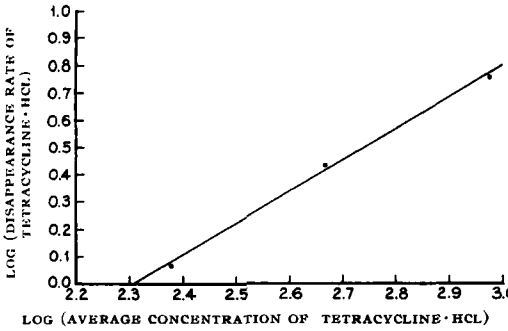

Fig. 1.—The rate of disappearance of tetracycline·HCl as a function of its average concentration at pH 4.0 and 23°C.

Three solutions of tetracycline were prepared in 0.10 *M* phosphate buffer at pH 4.0 and 23° at total tetracycline levels of 250, 500, and 1000 mcg./ml. Immediately after solution and 8.5 hours later, aliquots of the solutions were withdrawn and assayed for C·4 epimer. The experimental results are presented in Table I.

The reaction order for the C·4 epimerization was determined from the data in Table I using the differential method described by Laidler (6). In this method the logarithm of the rate of disappearance of tetracycline is plotted as a function of the logarithm of the average concentration of tetracycline as shown in Fig. 1. Such a plot should give a straight line, whose slope and intercept are equal to the reaction order and reaction rate constant, respectively.

In order to state that a reaction follows first-order reversible kinetics, (*a*) the slope of the line of the plot in Fig. 1 should be equal to 1.00, and (*b*) the experimental data when plotted according to Eq. 1 should give a straight line whose slope is equal to $k_1 + k_{-1}$.

Inspection of Fig. 1 indicates that the experimental observations appear to result in a straight line. The value obtained for the slope of the line was 1.134 with 95% confidence limits of 0.890 and 1.374. Since the value of 1.00 is within the confidence limits, the C·4 epimerization of tetracycline may be safely considered to satisfy condition (*a*) above for first-order reactions in the concentration range studied. The antilogarithm of the intercept gives a value of 2.443×10^{-3} hours^{-1} for the forward reaction rate constant. This value compares favorably with other values obtained in later experiments under the same conditions.

Determination of the Reaction Order for the Epimerization of 4-epi-Tetracycline to Tetracycline.—The experiment described above was repeated using 4-epi-tetracycline instead of tetracycline as the starting material. Three solutions of 4-epi-tetracycline were prepared in 0.10 *M* phosphate buffer at pH 4.0 and 23° at total tetracycline levels of 250, 500, and 1000 mcg./ml. Immediately after solution and at 7.5 hours later, aliquots of the solutions were withdrawn and assayed for C·4 epimer. The experimental results appear in Table II and are plotted in Fig. 2 in the same manner as for the previous experiment.

By the method outlined above, a value for the slope of 1.141 was obtained with 95% confidence limits of 0.823 and 1.449. Since the value of 1.00 is within the confidence limits, the epimerization of 4-epi-tetracycline to tetracycline may be considered to satisfy conditions (*a*) for first-order reactions in the concentration range studied. The antilogarithm of the intercept gives a value of 3.963×10^{-3} hours^{-1} for the backward reaction rate constant for the epimerization of tetracycline to 4-epi-tetracycline. This value compares favorably with

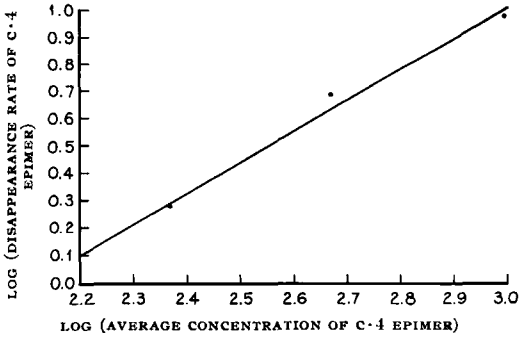

Fig. 2.—The rate of disappearance of C·4 epimer as a function of its average concentration at pH 4.0 and 23°.

TABLE II.—EXPERIMENTAL DISAPPEARANCE RATES AND AVERAGE CONCENTRATIONS FOR 4-EPI-TETRACYCLINE DURING FIRST 7.5 HOURS OF EPIMERIZATION

Initial Concn. of Tetracycline and C·4 Epimer	C·4 Epimer, Initial %	C·4 Epimer after 7.5 Hr., %	Rate of Disappearance $-\frac{\Delta(\text{C·4 Epimer})}{\Delta t}$	Av. Concn. of C·4 Epimer During First 7.5 Hr. of Reaction
250 mcg./ml.	96.62	90.86	1.920 mcg./ml.-hr.	234.4 mcg./ml.
500 mcg./ml.	96.62	89.43	4.793 mcg./ml.-hr.	465.1 mcg./ml.
1,000 mcg./ml.	96.62	89.67	9.267 mcg./ml.-hr.	931.5 mcg./ml.

TABLE III.—REACTION RATE CONSTANTS AS A FUNCTION OF PHOSPHATE BUFFER CONCENTRATION

Molar Concn. of Phosphate Buffer, pH 4.0 at 23°C.	Forward Reaction Rate Constant, $k_1 \times 10^3$, hr.$^{-1}$	Backward Reaction Rate Constant, $k_{-1} \times 10^3$, hr.$^{-1}$
0.01	0.406 (0.294–0.518)	0.662 (0.550–0.774)
0.10	2.13 (2.02–2.24)	3.47 (3.36–3.59)
1.00	15.5 (14.9–16.1)	25.3 (24.7–25.9)

TABLE IV.—REACTION RATE CONSTANTS AS A FUNCTION OF PHOSPHATE BUFFER CONCENTRATION

Molar Concn. of Phosphate Buffer, pH 4.0 at 23°C.	Forward Reaction Rate Constant, $k_1 \times 10^3$, hr.$^{-1}$	Backward Reaction Rate Constant, $k_{-1} \times 10^3$, hr.$^{-1}$
0.01	0.680 (0.670–0.690)	1.11 (1.10–1.12)
0.10	2.48 (1.83–3.13)	4.05 (3.40–4.70)
1.00	15.7 (6.71–24.6)	25.5 (16.6–34.5)

subsequent values obtained in later experiments under identical conditions.

The second condition for first-order reactions is demonstrated in the following experiments.

Effect of Phosphate Ion Concentration on the Epimerization of Tetracycline to 4-epi-Tetracycline.—Three solutions of tetracycline were prepared at a total tetracycline level of 1500 mcg./ml. in 0.01, 0.10, and 1.00 *M* phosphate buffer at pH 4.0 and 23°. Aliquots of the solutions were withdrawn periodically and assayed for C·4 epimer to determine the reaction rate constants under these conditions. Table III presents the reaction rate constants with their 95% confidence limits in parentheses.

Figure 3 shows that when the reaction rate constants are plotted on log-log paper as a function of the molarity of the phosphate buffer, a linear relationship is obtained which may be represented by

$$k_1 \times 10^3 = 22.5\,(M)^{0.791}$$
$$k_{-1} \times 10^3 = 23.9\,(M)^{0.791}$$

where M is the molarity of the phosphate buffer. The 95% confidence limits for the exponent were 0.728 and 0.854. Since the confidence limits do not include zero, the phosphate ion concentration exerted a significant effect on the rate of epimerization of tetracycline to its C·4 epimer at pH 4.0.

The above experiment was repeated using 4-epitetracycline as the principal starting material to determine the reaction rate constants under these conditions. Table IV presents the reaction rate constants with their 95% confidence limits in parentheses.

Using the reaction rate constants presented in Tables III and IV, one can construct a family of curves showing the C·4 epimerization as a function of time in phosphate buffer starting with either member of the epimeric pair. Such a family of curves is presented in Fig. 4. This figure shows that the equilibrium between epimeric pairs may be approached from either direction.

The rate constants in this experiment were determined by the method described earlier in this paper. When the experimental data from this and subsequent experiments were plotted according to Eq. 1, a straight line resulted, thereby satisfying the second condition for a first-order reversible reaction.

Effect of Citrate Ion Concentration on Epimerization of Tetracycline to 4-epi-Tetracycline.—The previous experiment was repeated using citrate buffers at pH 4.0 and 23° in the place of phosphate buffers. Aliquots of the solutions were withdrawn frequently and assayed for C·4 epimer to determine the reaction rate constants under these conditions. Table V summarizes the reaction rate constants with the 95% confidence limits in parentheses.

The reaction rate constants when plotted on log-log paper as a function of citrate molarity resulted in straight lines which may be represented by

$$k_1 \times 10^3 = 16.3\,(M)^{0.723}$$
$$k_{-1} \times 10^3 = 26.7\,(M)^{0.723}$$

where M is the molarity of the citrate buffer. The 95% confidence limits for the exponent are 0.714

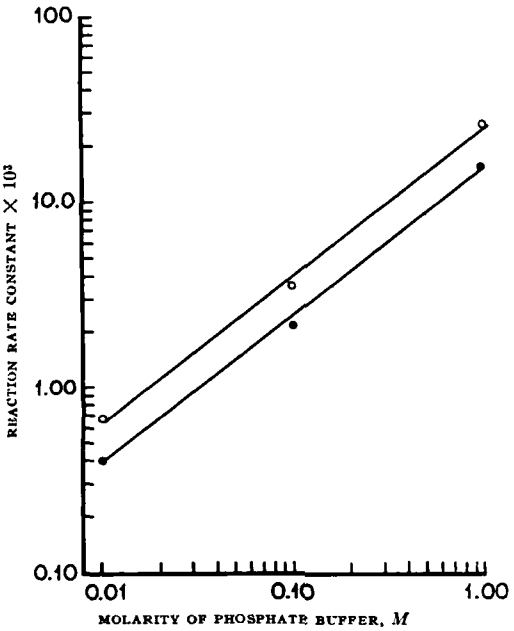

Fig. 3.—Reaction rate constants in phosphate buffers of various molarities at pH 4.0 and 23°: ●—forward reaction rate constant, ○—backward reaction rate constant.

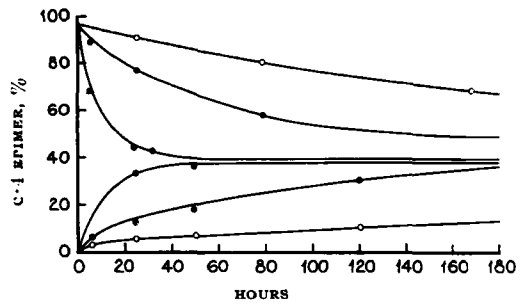

Fig. 4.—Equilibrium of tetracycline and its C·4 epimer in phosphate buffers of various molarities at 23°: ●—1.00*M* phosphate, pH, 4.0; ⊙—0.10*M* phosphate, pH 4.0; ○—0.01*M* phosphate, pH 4.0.

TABLE V.—REACTION RATE CONSTANTS AS A FUNCTION OF CITRATE BUFFER CONCENTRATION

Molar Concn. of Citrate Buffer, pH 4.0 at 23°C.	Forward Reaction Rate Constant, $k_1 \times 10^3$, hr.$^{-1}$	Backward Reaction Rate Constant, $k_{-1} \times 10^3$, hr.$^{-1}$
0.01	0.586 (0.439–0.733)	0.956 (0.717–1.195)
0.10	3.09 (2.23–3.87)	5.05 (3.79–6.31)
1.00	16.3 (12.3–20.4)	26.7 (20.0–33.3)

TABLE VI.—EFFECT OF TEMPERATURE ON THE C·4 EPIMERIZATION OF TETRACYCLINE

Temp., °C.	Forward Reaction Rate Constant, $k_1 \times 10^3$, hr.$^{-1}$	Backward Reaction Rate Constant, $k_{-1} \times 10^3$, hr.$^{-1}$
4	0.193 (0.165–0.221)	0.316 (0.288–0.344)
23	2.61 (2.20–3.02)	4.26 (3.85–4.67)
37	8.96 (6.86–11.0)	14.6 (12.5–16.7)
42	15.4 (8.62–2.22)	25.0 (18.2–31.8)
56	78.5 (20.5–178.0)	128.2 (29.2–227.0)

and 0.732. Since the confidence limits do not include zero, the citrate ion concentration exerted a significant effect on the rate of epimerization of tetracycline to its C·4 epimer at pH 4.0.

Rate of Epimerization of Tetracycline in Water.—A solution of tetracycline was prepared in distilled water at a total tetracycline level of 1500 mcg./ml. The solution was titrated to pH 4.0 with 0.1 *N* NaOH and allowed to epimerize at 23°. Aliquots of the solution were withdrawn periodically and assayed for C·4 epimer. During the aging of the unbuffered solution, the pH gradually fell from 4.0 to 3.5 in the first 10 days after which it remained constant at 3.5 for the remainder of the reaction. The reaction rate constants for this condition (with the 95% confidence limits in parentheses) can thus be summarized.

Forward Reaction-Rate Constants, $k_1 \times 10^3$, hr.$^{-1}$	Backward Reaction-Rate Constant, $k_{-1} \times 10^3$, hr.$^{-1}$
0.234 (0.014–0.454)	0.381 (0.161–0.601)

A comparison of the epimerization rates of tetracycline in 1.00 *M* phosphate and 1.0 *M* citrate buffer with that observed in distilled water under closely similar conditions of temperature and pH reveals that the epimerization rate in 1.00 *M* phosphate or citrate buffers proceeds approximately 70 times more rapidly than in distilled water.

Effect of Temperature on Epimerization of Tetracycline.—Solutions of tetracycline were prepared at a total tetracycline level of 1500 mcg./ml. in 0.10 *M* phosphate buffer at pH 4.0 that had been previously equilibrated at 4, 23, 37, 42, and 56°. Aliquots of the solutions were withdrawn frequently and assayed for C·4 epimer. Table VI presents the reaction rate constants with their 95% confidence limits in parentheses.

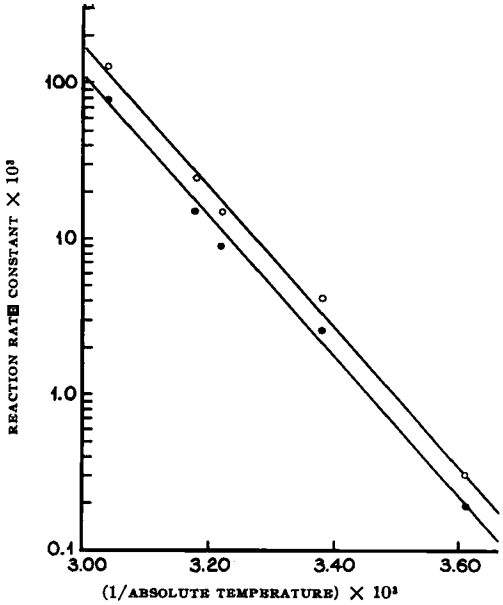

Fig. 5.—Arrhenius plot for the forward and backward reaction rates for the C·4 epimerization of tetracycline: ○—backward rate, ●—forward rate.

The above reaction rate constants were then plotted to give the Arrhenius plot presented in Fig. 5. Inspection of the figure reveals that the reaction rate constants fall rather closely on a straight line, the slope of which is equal to $-E/2.303\ R$ where E is the apparent energy of activation, and R is the gas constant, 1.986 calories per degree-mole.

The slope, activation energy, and frequency factors for the epimerization of tetracycline are presented in Table VII. The energy of activation and frequency factors from the Arrhenius plot have been obtained by a weighted regression analysis described by McBride and Villars (7). A weighted regression was used (*a*) to prevent equal weighting of rate constants determined with high precision with those obtained with low precision and (*b*) to compensate for the unequal weighting introduced by using the logarithm of the rate constant instead of the rate constant directly. McBride and Villars have shown that the above objections can be eliminated if the rate constants are weighted by a quantity directly proportional to the square of the rate constant and inversely proportional to its variance. The necessity for performing a weighted regression analysis can be seen from Table VI. At 56° the epimerization proceeded so rapidly that only three assays could be performed before the reaction was essentially complete. As a result, the reaction rate constant is estimated with low precision as indicated by the wide confidence interval. A weighted regression is used so that a rate constant determined

TABLE VII.—ACTIVATION ENERGY AND FREQUENCY FACTORS FOR THE C·4 EPIMERIZATION OF TETRACYCLINE

Reaction	Slope of Arrhenius Plot, 95% Confidence Limits	Activation Energy in Calories per Mole, 95% Confidence Limits	Frequency Factor, hr.$^{-1}$
Forward	−4.452 (−3.74)–(−5.16)	20,400 (17,100–23,600)	2.69×10^{15}
Backward	−4.452 (−3.89)–(−5.11)	20,400 (17,800–22,900)	3.61×10^{15}

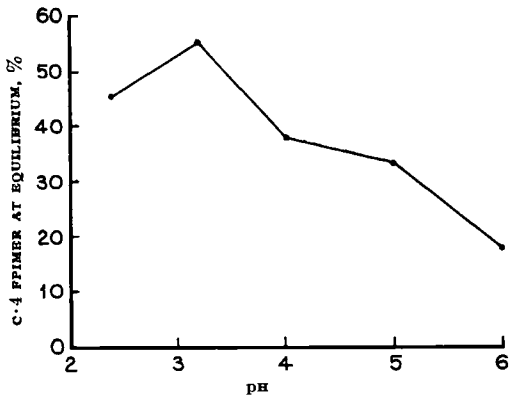

Fig. 6—Equilibrium concentrations of C-4 epimer as a function of pH.

with low precision is not weighted equally with one estimated with high precision.

Equilibrium Levels of C-4 Epimer at Various pH Levels.—The equilibrium concentrations of C-4 epimer and the rate at which equilibrium is approached were determined as a function of pH by aging solutions containing a total tetracycline level of 1500 mcg./ml. in 0.10 *M* phosphate buffer at 23° until the C-4 epimer level did not change with additional time. This concentration is considered to be the equilibrium concentration and is plotted in Fig. 6 as a function of pH. The equilibrium values employed in estimating reaction rate constants in this study are those presented in Fig. 6.

Inspection of Fig. 6 indicates that the equilibrium concentration of C-4 epimer is a function of the pH of the solution. Of the five pH levels studied, the maximum C-4 epimer concentration at equilibrium is 55% and occurs at pH 3.2.

The rate at which the epimerization occurred

TABLE VIII.—SUM OF FORWARD AND BACKWARD REACTION RATE CONSTANTS $[(k_1 + k_{-1}) \times 10^3]$ FOR TETRACYCLINE AS A FUNCTION OF pH (95% CONFIDENCE LIMITS)

pH	Rate Constants
2.4	7.45 (7.31–7.59)
3.2	8.76 (8.62–8.91)
4.0	8.90 (8.64–9.15)
5.0	8.71 (8.59–8.89)
6.0	5.39 (4.95–5.82)

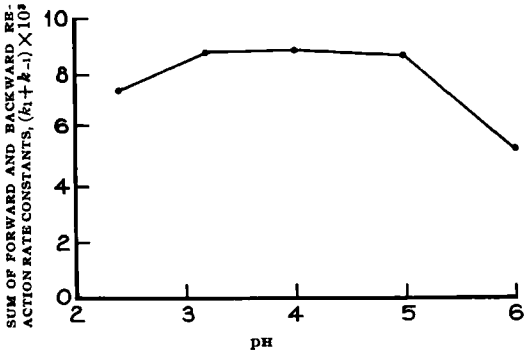

Fig. 7.—The sum of the forward and backward rate constants as a function of pH.

was also measured and is presented in Table VIII and Fig. 7. Inspection of the table and figure reveals that at pH 3.2, 4.0, and 5.0, the rates are essentially identical. As the pH is increased or decreased from the above pH range, the reaction rate declines.

SUMMARY

The C-4 epimerization of tetracycline to 4-epitetracycline follows first-order reversible kinetics at pH 4.0 in phosphate buffer. At pH 4.0 phosphate and citrate ions are effective in catalyzing the rate at which the equilibrium C-4 epimer concentration is approached.

The apparent energy of activation for the C-4 epimerization is approximately 20 kilocalories/gram-mole at pH 4.0 in phosphate buffer. The equilibrium concentration and the rate at which equilibrium is approached are dependent on the pH of the solution.

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Powdered Particle Interactions: Suspension Flocculation and Caking I

By ROBERT G. WILSON and BERNARD ECANOW

The phenomena of caking and of flocculation in a sulfamerazine suspension system have been reported in the literature. The concept of zeta potential was used in measuring and explaining the effects obtained. Several questions have been raised with regard to this prior work, and both caking and flocculation have been examined and explained in terms of other mechanisms. Flocculation in the sulfamerazine suspension system is principally due to the chemical reaction that takes place between dioctyl sodium sulfosuccinate anions and the trivalent cations of the flocculating agent. Relatively strong bonding forces develop between the suspended particles, and floccules are formed. Caking of the sulfamerazine suspension system is seen when the particles settle to a dense sediment before bonds can be established between the closely-packed particles.

HAINES AND MARTIN have recently reported on the phenomenon of flocculation (1–3). They have shown that relationships exist between zeta potential, flocculation, and caking tendencies for the particular suspension systems under study. A careful appraisal of their work has raised several questions and has prompted this laboratory to engage in additional research on the same systems. Initially, our attention has been directed toward the sulfamerazine suspension system on which Haines and Martin worked.

Fischer (4) has said that the electrostatic charge carried by dispersed particles is an important factor in the stability of an aqueous suspension. The charge originates by ionization of the surface or by adsorption of ions from the solution. Martin (5) has said that colloidal particles, as a result of their large surface areas and great surface forces, tend to adsorb ions from the medium and become positively or negatively charged. Fischer and Martin, in their separate works, go on to refer to the distribution of other ions in the form of an ionic atmosphere surrounding the charged suspended particle. Adamson (6) has presented a graphical picture of this concept by plotting electrical potential against distance from the surface of the particle. He states that a particle surface charge may be inherent or may be due to an adsorbed desolvated layer of ions. As we move away from the particle surface, we pass through the "stern layer," which is composed of adsorbed solvated ions surrounding the particle and tightly held to its surface. Moving beyond the stern layer, we enter the "diffuse gouy layer," where a density of ionic charge has gathered in response to the underlying charges on the particle.

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If an electrical field is imposed upon a colloidal system containing electrostatically charged particles, the charged particles will tend to move toward one or the other of the poles. Where other ions are present in the suspension medium, an individual particle as it moves will carry its adsorbed ions (stern layer) and a portion of its ionic atmosphere (diffuse gouy layer) along with it. A region of shear will be created between those ions moving with the particle, and those left behind to move independent of the particle. The electrical potential at this shear layer has been defined as the zeta potential (6). Zeta potential may be measured by observing the movement of a charged particle in a known electrical field or by measuring the magnitude of the electrical potential induced by the movement of the charged particle. The proper application of the concept of zeta potential has served to explain many of the phenomena exhibited by colloidal suspensions.

Fischer (7) has described the coagulation of colloidally dispersed charged particles in terms of the following hypothetical mechanism. Uncharged particles, when brought close together, adhere because of van der Waals attractive forces. In a stable suspension, where the particles are similarly charged, electrostatic repulsive forces predominate and the particles are prevented from adhering since the electrostatic forces are of longer range than the van der Waals forces. The magnitude of the repulsion is gauged roughly by the zeta potential. If the zeta potential is sufficiently high, the charges constitute a potential barrier and the colloidal system is stable. If the zeta potential is caused to decrease, as by the addition of an electrolyte, then the particles may have sufficient kinetic energy to cross the barrier and come within range of the van der Waals attractive forces. The

particles then adhere. The zeta potential corresponding to this limit is termed the critical potential.

Abramson (8) has discussed the work of Burton, who used potassium sulfate to coagulate a copper sol. With other conditions held constant, Burton modified the zeta potential on the copper particles by the addition of electrolyte. He reported his data in terms of electrophoretic velocity of the particles *versus* time for coagulation to occur, which is directly comparable to zeta potential *versus* the time for coagulation to occur. When enough potassium sulfate had been added to reduce the electrophoretic velocity to zero (a zeta potential of zero), it took 0.04 days (just under 60 minutes) for coagulation to occur. Burton thus demonstrated that (for this system) flocculation following the neutralization of zeta potential was a slow process. The rate of coagulation was dependent on the frequency of random collisions of the copper particles in the course of their Brownian movement. This seems to correlate with Fischer's thoughts, as reviewed in the preceding paragraph. With random Brownian movement, the particles may at times have sufficient kinetic energy and be properly oriented in their motion so that they will approach close enough to one another to be held by the van der Waals forces.

Zeta potential has thus been linked with colloidal systems and with the coagulation mechanism for a colloidal system.

Haines and Martin (1–3) worked with a sulfamerazine suspension system. They used a modified Northrop-Kunitz–type electrophoresis assembly and converted their electrophoretic velocity data to zeta potential values. Their summary (2) stated that the electrophoretic velocity in a nonflocculated sulfamerazine suspension was observed in a microscope electrophoresis apparatus and was found to be high because of the predominance of a negative charge on the particle. Electrical discharge of the particle by the addition of aluminum ions, which were opposite in charge to the particle, lowered the zeta potential and brought about flocculation of the particle, thereby reducing or eliminating the tendency to cake.

The ideas of Fischer (7) as given earlier in this paper, seem to have been confirmed by the findings of Haines and Martin. An important discrepancy, from the standpoint of this laboratory, is that Fischer had been discussing colloidal systems; Haines and Martin were working with particles larger than those normally considered to be within the colloidal range. Fischer (9) comments that the limits on colloidal particles have been variously stated, but sizes between 1 μ and 1 mμ encompass the usual extremes. Martin (10) gives the colloidal range as from 10 Å. up to 0.5 μ. The sulfamerazine particles used by Haines and Martin were in the 2 to 10-μ range and could be clearly seen under the microscope. Hauser (11) has said that colloidal dispersions are detectable only with the ultramicroscope, while coarse dispersions are microscopically resolvable. However, he adds that no sharp boundary exists between coarse dispersions, colloidal solutions, and true solutions. Hauser further states (12) that the principal differences between general and special types of disperse heterogeneous systems are the result of the appearance of special properties as the boundary surfaces are increased.

Among such special properties would be the van der Waals forces. Although they exist at the surface of all solid particles, they would be effective in bonding only when the solid was in an extremely fine state of subdivision, as for example in Burton's colloidal copper sol.

It is true that Kruyt (18), has referred to the theoretical existence of long-range London–van der Waals forces in connection with hydrophobic suspensions in the 2 to 5–μ range. However, he adds (19) that this kind of flocculation has not yet been verified experimentally. The sulfamerazine suspension under study is not a hydrophobic system, nor is it within the generally accepted colloidal range. It seems inappropriate to ascribe flocculation in such a system to the action of van der Waals forces following a neutralization of particle surface charge without experimental justification for such an assumption.

Initial work at this laboratory has shown that the flocculation of the sulfamerazine system by aluminum ions was virtually instantaneous on mixing. Burton (7) had been successful in applying zeta potential measurements to his copper sol only because the coagulation was slow to take place. The system was such that he had time to measure the zeta potential of the particles before they were lost to the coagulum. Haines and Martin were not so fortunate, and this has cast additional doubt on the use of zeta potential as a parameter for the flocculation of the sulfamerazine suspension system. They had calculated zeta potential from particle velocity data obtained in the electrophoretic cell and had assumed that cessation of particle movement was due to a neutralization of zeta potential. This does not necessarily follow, for the cessation of movement could be more aptly

explained by the flocculation phenomenon itself. Haines and Martin have indicated this to be the case, for in their discussion (2) they have said that the addition of flocculating agent caused the zeta potential to decrease, and at point B, the particles were observed in the microscope of the electrophoresis apparatus to exhibit maximum flocculation.

Two factors thus tend to negate the concept of a neutralization of zeta potential, followed by flocculation under the influence of van der Waals forces:

The particles are larger than those normally considered to be colloidal, and the effectiveness of inter-particulate van der Waals forces has not been demonstrated for suspensions of hydrophilic particles in this size range (2–10 μ).

When sufficient aluminum ions are present, flocculation is virtually immediate on mixing. Thus flocculation occurs before the zeta potential can be measured on the individual particles.

It seems desirable that an alternative explanation be given for the flocculation of a sulfamerazine suspension by aluminum ions. A word on the meaning of terms may be of assistance at this point. Fischer and Gans, in their chapter in Alexander (13), have defined the words "flocculation" and "deflocculation" as follows:

Flocculation—The formation of clusters of particles separable by a relatively weak mechanical force or by a change, as in chemical composition, at the interface between the particles and the suspending phase. Surface-active agents are often useful in reducing the extent of flocculation.

Deflocculation—The state of dispersion of a solid in a liquid in which each solid particle remains geometrically independent and unassociated with adjacent particles.

In an initial investigation, 2% of sulfamerazine was suspended in distilled water. A highly flocculated, bulky suspension was obtained. The uppermost curve of Fig. 1 serves to illustrate this fact. This suspension was then contrasted with a 2% suspension containing 0.010 moles per L. of aluminum chloride. There was no observable difference between the two suspensions. Both were equally flocculated according to the definition given by Fischer and Gans. It should be clearly recognized that aluminum ions have no effect on a simple sulfamerazine suspension.

Haines and Martin used dioctyl sodium sulfosuccinate (DSS)[1] as a wetting agent for the sulfamerazine to reduce the hydrophobic nature of the sulfamerazine particles. This was a typical deflocculation reaction as defined above. When aluminum ions were added to such a sulfamerazine suspension system, a return to a flocculated condition was obtained. It would appear that the DSS was playing an integral part in the flocculation phenomenon, even though Haines and Martin made it a point to say (1) that the addition of the surface-active agent was found to have little or no effect on the recorded observations.

[1] Marketed as Aerosol-OT by American Cyanamid Co., New York, N. Y.

EXPERIMENTAL

Suspension System—During the current studies, the suspension concentration was held at the 2% solids level. Haines and Martin had shown that higher concentrations brought on the problem of hindered settling, and lower concentrations increased the errors inherent in the reading of flocculation volumes. DSS concentrations were varied from 0.001% up to 0.25%. To prepare the suspensions, the sulfamerazine powder was weighed, transferred to a glass mortar, and triturated with the required amount of a concentrated wetting-agent solution until a smooth slurry was obtained. The slurry was then transferred, with rinsing of the mortar, to a cylindrical graduate and distilled water was added to bring the volume to the proper level. Where flocculating agents were under study, they were added in the form of a concentrated solution during the adjustment of the final volume of the suspension. The graduate was then stoppered, inverted, and agitated to insure both thorough mixing and the uniform initial suspension of the solid.

Settling Data.—The methods used by Ward and Kammermeyer (14) were utilized in obtaining, and in some cases in reporting, the sedimentation data. It becomes necessary to qualify the above statement, for the sulfamerazine systems are not consistent in their settling characteristics. In some of the flocculation reactions, as much as 25% of the flocculated material floated to the surface of the medium rather than sedimenting as would have been expected. The total volume of flocculated material, summing the volume of both floating and sedimented floccules, was reported and used in comparing one system to another.

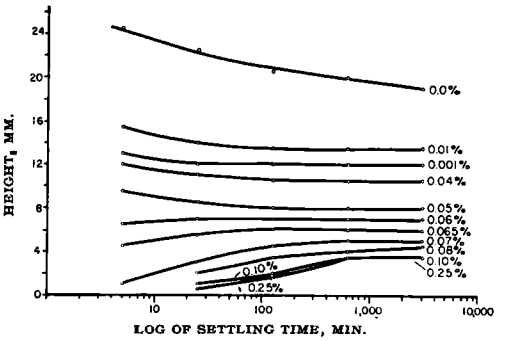

Fig. 1.—Height of sediment *vs.* log of settling time for 2% sulfamerazine suspended in various concentrations of dioctyl sodium sulfosuccinate solution.

Equipment.—Settling data was obtained in 100-ml. glass-stoppered cylindrical graduates. Suspension heights or flocculation volumes could be measured within ±0.5 ml. accuracy, which was more than sufficient to point out the differences between the various suspension formulations.

Materials.—A commercial grade of sulfamerazine U.S.P. as supplied by Merck Sharp & Dohme was used. The suspending medium was distilled water containing the wetting agent dioctyl sodium sulfosuccinate (100%) as supplied by American Cyanamid Co. The two flocculating agents, aluminum chloride ($AlCl_3 \cdot 6\ H_2O$) and ferric chloride ($FeCl_3 \cdot 6\ H_2O$), were reagent grade chemicals as supplied by Merck & Co.

RESULTS

Figure 1 presents the data on settling rates for 2% sulfamerazine suspensions containing various concentrations of the wetting agent. The critical micelle concentration for DSS has been given as approximately 0.07%, at 25° (5 to 15 seconds) (15).

At DSS levels above the critical micelle concentration, the sulfamerazine was held in a finely divided state and settled slowly to a dense sediment with definite caking tendencies. The sediment built up slowly at the bottom of the container and the supernatant liquid very slowly cleared to denote the completion of sedimentation. Above 0.1% DSS, changes in the wetting agent concentration gave practically no change in the settling rate curve.

Below the critical micelle concentration, the suspended sulfamerazine particles clumped and the flocculated material slowly compacted to its final volume. In this range of wetting-agent concentration, the suspension began to exhibit the hydrophobic properties of the basic sulfamerazine particle. As wetting-agent concentration decreased, increasing amounts of the solid tended to float to the surface until, in the absence of wetting agent, as much as 25% of the sulfamerazine floated to the surface.

Figure 2 illustrates the action of aluminum chloride as a flocculating agent for a 2% sulfamerazine suspension in 0.25% DSS solution. Small quantities of aluminum chloride had no apparent effect upon the suspension, other than to give the supernatant liquid a slight opalescence. The suspended particles acted just as they would at wetting-agent concentrations above the point of micelle formation.

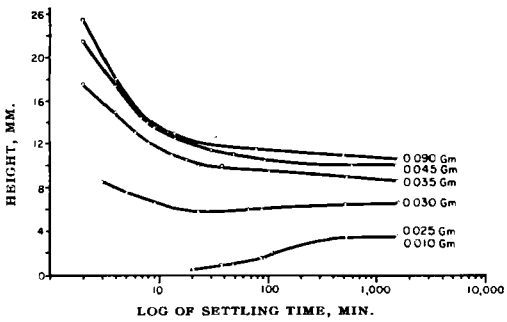

Fig. 2.—Height of sediment *vs.* log of settling time for 2% sulfamerazine suspended in 0.25% dioctyl sodium sulfosuccinate solution. Various amounts of aluminum chloride were added to cause flocculation.

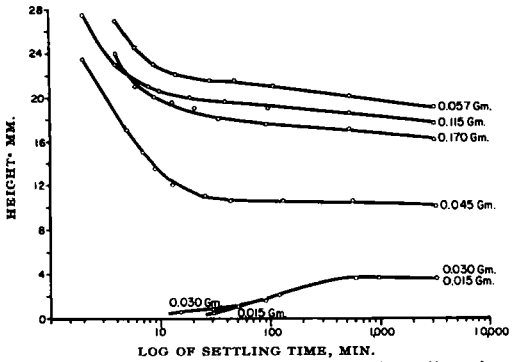

Fig. 3.—Height of sediment *vs.* log of settling time for 2% sulfamerazine suspended in 0.25% dioctyl sodium sulfosuccinate solution. Various amounts of ferric chloride were added to cause flocculation.

As aluminum chloride was added in larger amounts, the sulfamerazine particles grouped as clumps or floccules, and separated from the medium both as a loose, bulky precipitate, and as a floating layer of clumped material. At these higher levels of aluminum chloride addition, the supernatant liquid was clear and colorless.

Figure 3 charts the effect of ferric ion on a sulfamerazine suspension in 0.25% DSS solution. Small additions of ferric chloride had no apparent effect upon the suspension. The supernatant liquid became slightly opalescent, but the suspension performed just as it would at wetting-agent levels above the critical micelle concentration.

At the level of 0.045 Gm. ferric chloride per 100 ml., a definite soft floc was obtained. It sedimented, leaving an opalescent solution that contained no floating particles of sulfamerazine. The floc could be easily resuspended on shaking. When 0.057 Gm. ferric chloride per 100 ml. was added, the uppermost curve on the graph was obtained. At this level, the most fluffy and voluminous floc was formed. Half of the floccules floated to the surface, while the balance slowly settled out to leave a markedly opalescent supernatant liquid.

At the higher levels of ferric chloride addition, a slightly more compacted floc was obtained. It was of interest to note that the floc took on the color of the ferric ion, while the supernatant liquid became clear and essentially colorless.

The opalescence noted in many of the above preparations indicated a possible chemical interaction between the DSS anion and the trivalent cation. When a dilute solution of aluminum chloride was added (slowly and with stirring) to an 0.25% DSS solution, an opalescence was seen. As more aluminum ion was added, a milky, colloidal suspension formed and promptly coagulated to a few floating wax-like particles. These particles were filtered off, thoroughly washed with dilute ammonium chloride solution, dried, and a weighed amount ignited to a residue of aluminum oxide. The residue was 5.00% of the original weight.[2] Assuming the product had been an aluminum salt of dioctyl sulfosuccinic acid, the original compound was calculated to have the formula: $Al(C_{20}H_{37}O_7S)_2\ Cl \cdot xH_2O$. The above procedure was repeated

[2] Analytical data by Micro-Tek Laboratories, Skokie, Ill.

using ferric chloride as the precipitating agent. A milky, orange-colored colloidal suspension was formed, from which the organic material was extracted with ether. Upon evaporation of the ether, a viscous orange-brown residue was obtained. A weighed amount of the residue was ignited to ferric oxide and the weight of the oxide proved to be 7.24% of the original weight.[2] Assuming the residue was a ferric salt of dioctyl sulfosuccinic acid, it was calculated to have the following empirical formula: $Fe(C_{20}H_{37}O_7S)_2Cl \cdot xH_2O$.

Macroscopic observations on the various suspensions were correlated with microscopic studies. In those suspensions where the DSS was present in an amount above the critical micelle concentration, the sulfamerazine was present as discrete particles in a deflocculated condition. The particles were irregular, plate-like crystals of from 2 to 10 μ in size.

Where flocculating agents had been added in moderate amounts, the floccules were readily visible to the naked eye. On shaking, these relatively large flocculated masses were reduced in size, forming a finely divided suspension from which the original flocculated system was reformed within a few minutes. Microscopic observation indicated that there was some form of "adherence" between the sulfamerazine particles. The individual particles were grouped into small floccules, and these were further formed into loose, lacy masses. Higher concentrations of the flocculating agents gave floccules that were more granular in their macroscopic appearance. Microscopically, these floccules seemed to be larger in size and more compact than the flocculated masses obtained at lower levels of flocculating agent addition.

DISCUSSION

A 2% sulfamerazine suspension system has been used to study the phenomenon of flocculation. By itself, sulfamerazine in water is seen to give a highly flocculated suspension. If a small amount of an anionic wetting agent is introduced, some wetting occurs and the degree of flocculation is reduced. The suspension is further deflocculated as the concentration of wetting agent is increased until the critical micelle concentration has been exceeded. Above this level of wetting-agent concentration, the individual sulfamerazine particles have become coated by the anions of the wetting agent and are dispersed in the form of a completely deflocculated suspension.

An 0.25% DSS solution was selected for the further experimental work. The wetting-agent concentration was thus initially above the level of critical micelle concentration. As flocculating agent (aluminum or ferric ion) was added, there was no significant change in the state of the suspension until enough flocculating agent had been added to reduce the concentration of DSS anion below the level at which it could form micelles. From this point on, gradual addition of the flocculating agent gave increasing flocculation up to the maximum value obtained. Addition of agent beyond this point seemed to produce a slightly more compacted flocculation.

From the above, we can list several factors as being involved in the flocculation of this system: (*a*) the wetting agent concentration, (*b*) the neutralization or precipitation of wetting agent by the addition of flocculating agent, (*c*) other unknown factors, or (*d*) a combination of two or more of the above.

A chemical reaction has been shown to take place between aluminum or ferric ions and the dioctyl sulfosuccinate anion. If the sole effect of this reaction had been the neutralization of wetting-agent action, the flocculation curves would have coincided with those obtained by varying the amount of wetting agent in the suspension medium. This was certainly not the case with aluminum ions, and the divergence was even more marked when ferric ions were the flocculating agents. The chemical reaction thus appeared to be the most significant factor in the flocculation mechanism.

At the start of each experimental run, the sulfamerazine particles were coated with wetting-agent anions and the suspension was in a deflocculated condition. Each particle thus had a negative charge and was surrounded by its own ionic atmosphere. The general picture was that of hydrocarbon chains adsorbed to the surfaces of the particles with negative sulfonic acid radicals projecting into the surrounding medium. Aluminum ions were then added to the system, and they promptly reacted with DSS anions, whether these anions were adsorbed on particle surfaces or were available as ions in the suspension medium. Aluminum di-(dioctyl sulfosuccinate) chloride was formed as an insoluble product of the reaction. The sulfamerazine particles went into a state of flocculation which was modified by the presence of the aluminum compound.

The flocculation mechanism has been discussed, and it now remains for us to consider the phenomenon of caking in the sulfamerazine suspension system.

Haines and Martin (1) have said that when dispersed particles form into a compact mass at the bottom of the container, the suspension is said to have caked. They listed two types of structures that may be formed: the condensation or strongly bonded type, and the coagulation or weakly bonded type. Examples of both types were given. The coagulation or weakly bonded type of interaction was said to be due to the adhesion of particles by molecular interaction at points of contact between the particles. The weak bonds that are formed are due to a squeezing out of the dispersion medium at the points of contact, as in syneresis. The resulting residual layer of the dispersion medium prevents a strong molecular interaction at the points of contact. The strength of the cake which is formed depends upon the number of particles occupying a given volume. The particles may orient themselves in the sediment and, under the influence of gravity, coagulate in an arrangement of closest packing. Various clays, such as bentonite, were said to exhibit this type of sediment.

Hauser (17) has also commented on such a system. He has said that where suspended anisodimensional particles are allowed to settle of their own accord, they will tend to pack themselves in such a way that a sediment of minimum free energy results. This means that the particles will line up with their larger surfaces opposite each other. The solvated layer surrounding each particle will act somewhat like a lubricant, permitting the particles to slide over each other until they have found their proper location. With further sedimentation, the solvated

layers of the first settled particles will become distorted or deformed. Hauser goes on to say that this deformation will result in a coalescence of the solvated layers, since this would also represent a shift toward a system having a lower level of free energy. The heavier the particles, the greater the deformation of the solvated layers and thus the greater the chance for their coalescence.

Overbeek's chapter in Kruyt (19) contains the following observation: "There are, however, great differences in the sediments of stable and flocculated suspensions. The stable sediments are very closely packed because in forming the sediment the particles retain their individual freedom and roll over each other until the closest packing is reached. The flocculated sediments are more loosely built because once the sedimenting particles touch each other they adhere in the haphazard way in which they first touched and are no longer able to proceed into a more favorable position."

In the course of this study, three sulfamerazine systems were under observation:

Powered sulfamerazine in water. This suspension system was highly flocculated, and there was no caking problem.

Powdered sulfamerazine suspended in a DSS solution. This system gave a deflocculated suspension that settled to a dense sediment with definite caking tendencies.

Powdered sulfamerazine in DSS solution to which aluminum ions were added as a flocculating agent. A highly flocculated suspension with no evidence of caking was obtained.

This portion of the work has confirmed the findings of Haines and Martin (1) that a suspension in a state of fine deflocculated particles leads to rather than obviates caking. It also echoes the findings of Fischer (16) that flocculated particles settle rapidly to high volumes, while deflocculated particles settle slowly to low relative volumes. In flocculated systems, it would appear that the bonding forces between particles are relatively large, for the particles are held together in the form of loose clumps. When these clumps settle out, the particles remain distributed throughout a large volume of space. They cannot pack closely against one another and are thus unable to form into a cake.

The caking of sulfamerazine suspended in DSS solution is an example of the weakly bonded type of interaction. As originally prepared, the sulfamerazine particles are coated with DSS anions and are suspended as independent, charged particles in the medium. Since the system is not colloidal, gravity acts to pull the particles down into a sediment of low relative volume. As the particles settle, the suspension medium is squeezed out and the solvated layers surrounding each particle come into close contact with one another. Under the continuing influence of gravity, there is a coalescence of the solvated layers and a type of soft cake is formed. The cake can be returned to its original finely divided suspended state by shifting the particles enough to allow some of the suspension medium to come between them. The individual solvated layers are thus restored, and the particles can be resuspended in the deflocculated state. Aging studies are being conducted to see if the caking reaction becomes irreversible with time.

The concept of zeta potential does not provide us with a suitable picture of the sulfamerazine suspension systems. Attention should be focused upon the strength of bonding forces and the extent to which large areas of particle surfaces come into close contact with each other. As has been said, the interparticulate bonds in the flocculated system seem stronger and are more rapidly formed than are those in the deflocculated system. When the particles flocculate, bonding strengths are such that the particles are unable to settle to a closely packed sediment. For deflocculated particles, the bonding forces seem weak and are slow to establish themselves. The particles must settle and shift until large areas of surface are in close proximity to each other before the cake is formed. The relative strength of the bonds and the extent of contact between particle surfaces are thus the determining factors in the formation of a floc, a cake, or a densely packed sediment which shows no caking tendencies.

SUMMARY

The phenomena of caking and of flocculation in a sulfamerazine suspension system have been reported in the literature. The concept of zeta potential was used in measuring and explaining the effects obtained. Several questions have been raised with regard to this prior work, and both caking and flocculation have been examined and explained in terms of other mechanisms.

Flocculation in the sulfamerazine suspension system is principally due to the chemical reaction that takes place between dioctyl sodium sulfosuccinate anions and the trivalent cations of the flocculating agent. Relatively strong bonding forces develop between the suspended particles, and floccules are formed.

Caking of the sulfamerazine suspension system is seen when the particles settle to a dense sediment before bonds can be established between the closely packed particles.

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Absorption, Metabolism, and Excretion of the Semisynthetic Penicillin 6-(2-Ethoxy-1-naphthamido)penicillanic Acid (Nafcillin)

By SIDNEY S. WALKENSTEIN, ROBERT WISER, EDITH LEBOUTILLIER, CATHRYN GUDMUNDSEN, and HAZEL KIMMEL

Nafcillin, 6-(2-ethoxy-1-naphthamido)penicillanic acid, a semisynthetic penicillin effective against penicillin G-resistant staphylococci, was labeled with carbon-14, and its absorption, metabolism, and excretion studied in dogs and rats after oral and intramuscular administration. Maximum plasma levels were attained within 15 minutes of an intramuscular dose and within 2 hours of an oral dose, although detectable levels appeared in plasma within 30 minutes in the latter case. Low levels of activity persisted in plasma up to 8 days, although about one-third was excreted in feces and about one-sixth in urine within 48 hours. Thirty minutes after injection, the drug was found in all major organs and tissues examined. Within the first 6 hours, about half the carbon-14 excreted in urine was due to nafcillin, about 30 per cent was due to a metabolite of similar R_f to unchanged drug, and the remaining 20 per cent was divided equally between two other metabolites.

AMONG THE NEWER penicillins synthesized from 6-aminopenicillanic acid, 6-(2-ethoxy-1-naphthamido)penicillanic acid, nafcillin (Wy-3277), has been found effective *in vitro* (1) and *in vivo* (2) against penicillin G -resistant staphylococci.

To enable us to investigate the metabolism, absorption, and excretion of the drug more completely than was possible in a non-isotopic study (3), C^{14}-labeled nafcillin was employed.

METHODS

2-Ethoxy-1-naphthoic acid-carboxyl-C^{14} (I) was synthesized by C^{14}-carbonation of the Grignard reagent prepared from 7.5 Gm. (0.03 mole) of 2-ethoxy-1-bromonaphthalene in tetrahydrofuran. The carbonation was performed in a manner previously described (4). Radioactive carbon dioxide was generated from 5.9 Gm. (0.03 mole) $BaC^{14}O_3$ (10 mc.). The product was isolated by acidification of the reaction mixture to pH 2 with 10% sulfuric acid, separation of the organic layer, and evaporation of the tetrahydrofuran. The residue was treated with 10% sodium hydroxide and cyclohexane, warmed to 50°, and cooled, the aqueous layer removed and adjusted to pH 7 with 5 *N* sulfuric acid. This solution was heated to 70° and filtered. The warm filtrate was acidified to pH 2, cooled, the solid filtered off and dried at 60°, m.p. 141–145°. The yield was 4 Gm. (61%).

The preparation of labeled 6-(2-ethoxy-1-naphthamido)penicillanic acid and its sodium salt involve only slight modifications of procedures described in the patent literature (5–7). Four grams of I were diluted with 1.2 Gm. of non-labeled acid (0.024 mole total) for conversion to the acid chloride. Thionyl chloride (1.9 ml.) was added dropwise to a stirred mixture of acid, 0.1 ml. dimethylformamide, and 9 ml. of dichloromethane. After addition was complete, the mixture was refluxed for 1 hour. The excess thionyl chloride was removed and the dichloromethane solution of the acid chloride used for the reaction with 6-APA.

6-APA (5.1 Gm., 0.024 mole) and triethylamine (5.1 Gm., 0.05 mole) were combined in 24 ml. of dichloromethane, cooled, and treated with the solution of the acid chloride. The mixture was stirred for 1.5 hours and then adjusted to pH 2 with 1 *N* sulfuric acid. The dichloromethane layer was separated, washed with water, and carefully extracted with 24 ml. of 1 *N* sodium bicarbonate. The phases were separated and the dichloromethane extracted again with 10 ml. of 1 *N* sodium bicarbonate. The sodium bicarbonate extracts were combined, treated with 3 ml. of methyl isobutyl ketone and acidified to pH 2 with 1 *N* sulfuric acid. After standing a short time, the water was decanted from the gummy precipitate and 5 ml. of methyl isobutyl ketone was added to the residue. Stirring caused the product to crystallize. The solid was filtered off, washed with 1.7 ml. of cold methyl isobutyl ketone, 6.8 ml. of cold butanol, and finally with 17 ml. of cold ether. The yield of light tan product, air dried overnight (m.p. 138–141°) was 4.1 Gm. (41%). This material was suspended in 3.5 ml. of acetone to which 5.5 ml. of 2 *N* sodium 2-ethylhexanoate in butanol was added, and the mixture stirred until solution was complete. The solution was treated with Darco and filtered. After dilution with *n*-butanol (17.5 ml.), cooling and stirring promoted crystallization of the salt. The product was washed several times with ether and recrystallized by dissolving in a minimum volume of acetone and diluting with four volumes of absolute ethanol. The yield was 2.2 Gm. of sodium salt with a specific activity of 1210 disintegrations per mcg. A second batch of labeled material was obtained by concentrating the filtrate to incipient dryness, adding 2 Gm. of non-labeled nafcillin and recovering the salt (407 disintegrations per mcg.)

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as described above. Both samples of radioactive product were chromatographically pure and when assayed microbiologically found to correspond with nonradioactive nafcillin (200 penicillin G units per mg.). The salt is hygroscopic.

Samples of tissue, plasma, and urine, ranging in size from 50 to 200 mcg. were placed in counting vials containing 2 ml. of Hyamine-10X, 1 *M* solution in methanol (Packard Instrument Co.) and kept at 55° for 48 hours. Each sample was then treated with 100 μl. of 30% hydrogen peroxide, and after 10 minutes at room temperature, the samples were acidified with concentrated hydrochloric acid. To each sample, 2 ml. of absolute ethanol and 10 ml. of scintillator solution (5 Gm. of 2,5-diphenyloxazole plus 100 mg. of 1,4-bis-2-(5-phenyloxazolyl)benzene per liter of toluene) was then added. The vials were read in a liquid scintillation spectrometer (Packard Instrument Co.) at 2°.

Feces were converted to carbon dioxide by wet Van Slyke combustion and assayed as such in a Nuclear-Chicago Dynacon electrometer. Urine, plasma, and bile were submitted for microbiological assay.

C^{14}-Nafcillin was given orally in gelatin capsules and intramuscularly in 10% water solution to dogs at a dose of 10 mg. of sodium salt per Kg. body weight. Samples of urine, feces, and plasma (heparinized) were taken at various time intervals for 8 days from the six dogs in each group. The bile ducts of four other dogs were cannulated and bile and urine collected continuously for 6 hours while the animals were kept under sedation with sodium pentothal. At the end of the sixth hour, collection balloons were affixed to the cannulae and the sedation was ended. At 24 hours the dogs were sacrificed and the bile was removed from the collection balloons. Two of the animals had been given 10 mg. per Kg. orally in gelatin capsules prior to the operation and two were injected with a similar dose intramuscularly following cannulation of the bile ducts, a procedure which took 15 minutes.

For the study of the distribution in the tissues of rats the drug was given in aqueous solution orally (stomach tube) and intramuscularly at a dose of 23.6 mg. per Kg. In each group, three rats were sacrificed per time interval up to 48 hours and dissected for radioassay of tissues.

Ascending paper chromatograms of urine samples were made on Whatman No. 1 paper developed in butanol, acetic acid, water (4:1:5, upper phase) and in butanol, pyridine, water (45:25:20). After development, the chromatograms were radioautographed on NO Screen X-ray film and subsequently cut into 1/2-in. sections for assay in a thin-window flow counter, or assayed microbiologically by application to the surface of agar seeded with *Staphylococcus aureus* followed by incubation at 37° for 16 hours.

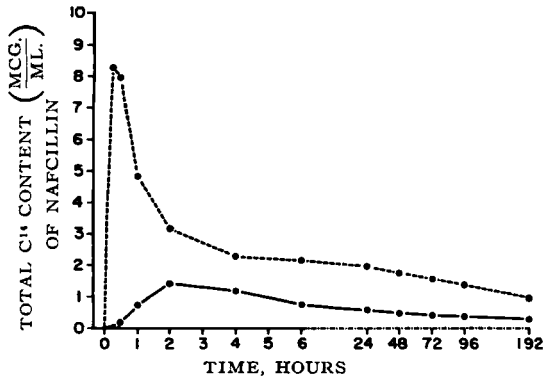

Fig. 1.—Plasma levels of C^{14} following a single dose of 10 mg. per Kg. C^{14}-nafcillin in dogs.

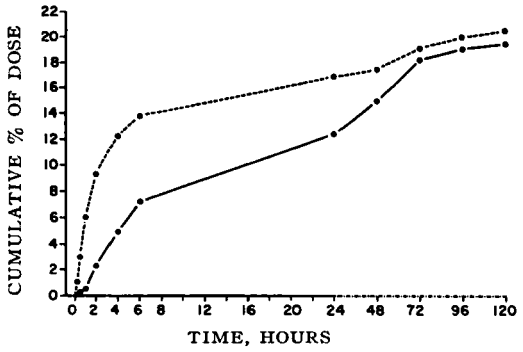

Fig. 2.—Urinary excretion of C^{14} following a single dose of 10 mg. per Kg. of C^{14}-nafcillin in dogs.

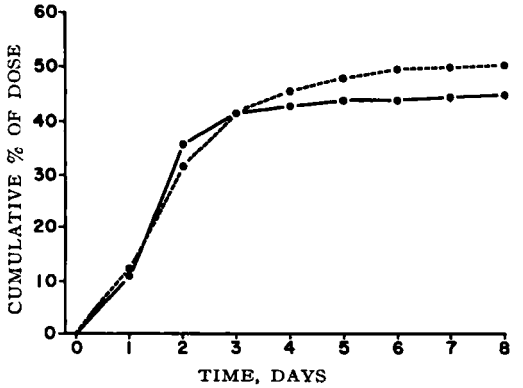

Fig. 3. Fecal excretion of C^{14} following a single dose of 10 mg. per Kg. of C^{14}-nafcillin in dogs.

RESULTS

Plasma Levels in Dogs.—Virtually all the radioactivity present in whole blood resided in the plasma fraction. Rapid absorption from the intramuscular injection site was indicated by a peak plasma level of C^{14} equivalent to 8.2 mcg. per ml. of nafcillin at 15 minutes, followed by a decline at a rate of about 2.5 mcg. per ml. per hour to the second hour (Fig. 1). After 8 days, the amount of radioactivity present in plasma amounted to the equivalent of approximately 1 mcg. per ml. Given by the oral route, the plasma level of C^{14} rose to about 1.4 mcg. per ml. by the second hour and then fell linearly to about 0.25 mcg. per ml. at the eighth day.

Urinary Excretion in Dogs.—Appreciable radioactivity amounting to an average of 1% of an intramuscular dose appeared in the urine within 15 minutes, 6% at 1 hour (Fig. 2). Following an oral dose, about 0.3% was excreted in the urine within the first hour. By the sixth hour 14% of the intra-

TABLE I.—C^{14}-TISSUE LEVELS[a] IN RATS AFTER A SINGLE INTRAMUSCULAR DOSE (23.6 MG./KG.) OF C^{14}-NAFCILLIN

Tissue	1/2 hr.	1 hr.	2 hr.	4 hr.	6 hr.	24 hr.	48 hr.
Plasma	17.5	10.4	1.3	0.6	0.45	0.32	0.27
Lung	6.7	5.8	0.4	0	0	0	0
Liver	145.9	243.6	37.8	11.6	5.9	0.99	0.7
Brain	0.3	0.3	0	0	0	0	0
Heart	5.0	2.2	0.2	0	0	0	0
Spleen	2.6	1.9	0.1	0	0	0	0
Kidney	97.5	58.4	4.9	0.5	0.62	0	0
Stomach	4.7	1.8	1.4	1.1	1.7	0	1.76
Muscle	1.5	1.7	0	1.1	0	0	0
Skin	4.0	2.5	0.9	0.7	0.1	0	0
Bladder	11.4	82.3	13.0	0.5	0.5	0	0.13
Cecal wall	3.8	2.1	3.7	166.5	752.2	112.5	153.0
Small intestine	289	795	587.3	375	101.3	5.9	16.9
Fat	2.0	0	1.4	0	0	0	0
Urine[b]	...	...	...	...	2.2%	11.5%	23.2%
Cecal contents[b]	0	0	0.17%	16.6%	33.3%	30 %	8.3%
Feces[b]	0	0	0	...	...	21.5%	36.2%

[a] Average values, three animals per group, expressed as equivalent amount of nafcillin in mcg./Gm. of tissue. [b] Total collection, expressed as per cent of dose.

muscular dose and 7% of the oral dose was excreted. However, by the third day, regardless of route of administration, roughly the same fraction of the dose of radioactivity, 18–19%, appeared in urine. After the third day, little additional radioactivity appeared and the total amounts excreted in the urine by the eighth day after intramuscular and oral dosing were 21 and 19.4%, respectively.

Fecal Excretion in Dogs.—Approximately the same amount of radioactivity was excreted in feces after both intramuscular and oral administration (Fig. 3), about 10% in the first 24 hours, 20–25% in the next 24 hours. The total by the eighth day amounted to almost 50% of the dose.

Distribution in Tissue of Rats.—Within 30 minutes of an intramuscular dose, radioactivity was found in all major organs and tissues (Table I). The highest concentrations appeared in small intestine, liver, and kidney. Since cecal wall was rinsed free of its contents prior to assay, the high values for this tissue indicate a fair degree of binding. The plasma level at 30 minutes, 17.5 mcg. per ml., was what one might anticipate from the results in dogs if one considers that the latter received half the dose given the rats. However, at 24 and 48 hours, the plasma levels were only one-eighth those of intramuscularly injected dogs. The relative amounts of radioactivity appearing in feces and urine correspond roughly with those of dog excreta, with a 2:1 ratio of fecal to urinary content.

Tissue levels following an oral dose of C^{14}-nafcillin in rats were lower than after an intramuscular dose (Table II). That these lowered values are partly the result of the slow rate of gastric emptying in the rat can be seen by the values for stomach which, at 4 hours, still contained an appreciable amount of the drug. The drug seems to be more poorly absorbed from the gut of the rat than from that of the dog (since plasma levels were lower than in dogs, though the dose was higher) and at 48 hours the rats had excreted only half as much radioactivity in the urine as had the dogs. Another indication of the poor absorption from the gut of the rat is the ratio of fecal to urinary excretion, about 10:1 (including cecal contents) in the oral-dosed rats compared with about 2:1 with the intramuscular-dosed rats. It will be recalled that the ratio was 2:1 in dogs for both routes of administration.

Metabolism.—It was shown in Fig. 2 that 14% of injected radioactivity was excreted in urine

TABLE II.—C^{14}-TISSUE LEVELS[a] IN RATS AFTER A SINGLE ORAL DOSE (23.6 MG./KG.) OF C^{14}-NAFCILLIN

Tissue	1/2 hr.	1 hr.	2 hr.	4 hr.	6 hr.	24 hr.	48 hr.
Plasma	0.57	0.71	0.75	0.30	0.15	0	0.08
Lung	0.76	0.10	1.82	0	0	0	0
Liver	19.5	21.5	19.8	8.77	5.55	2.57	0.11
Brain	0.08	0	0	0	0	0	0
Heart	0	0	0	0	0	0	0
Spleen	0.27	0	0	0	0	0	0
Kidney	1.06	1.31	1.53	0.42	0.10	0	0
Stomach	693	759	426	140	12	0.57	1.0
Muscle	0.10	0.26	1.32	0	0	0	0
Skin	0	0	0	0	0	0	0
Bladder	0.98	1.13	0.28	1.30	0.15	0.06	0
Cecal wall	0.16	0	18.22	514	926	222	111
Small intestine	499	588	655	183	72.3	3.88	1.86
Fat	0.06	0.12	0.25	0	0	0	0
Urine[b]	0.03%	0.16%	0.60%	1.47%	1.3%	2.8%	6.7%
Cecal contents[b]	0	0	2.7 %	32.9 %	40.5%	32.0%	9.0%
Feces[b]	...	...	...	...	...	12.7%	58.2%

[a] Average values, three animals per group, expressed as equivalent amount of nafcillin in mg./Gm. of tissue. [b] Total collection expressed as per cent of dose.

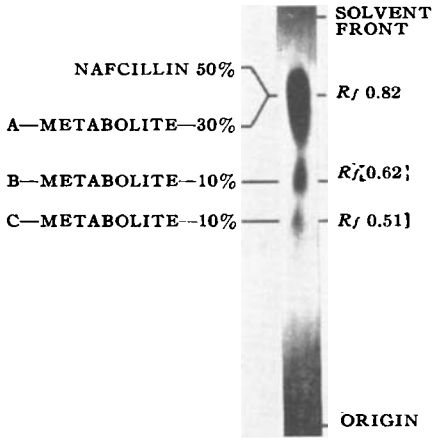

Fig. 4.—Ascending radioautograph of paper chromatogram of metabolites of nafcillin in urine. Solvent: butanol, glacial acetic acid, water (4:1:5) (upper phase).

TABLE III.—$\left[\frac{\text{MICROBIOLOGICAL}}{\text{RADIO-}}\right]$ ACTIVITY OF DOG URINE AND PLASMA SAMPLES[a] AFTER AN INTRAMUSCULAR DOSE

Time, hr.	Urine, %	Plasma, %
1/4	64	30
1/2	50	40
1	62	20
2	57	5
4	67	0
6	53	0
24	14	0

[a] Values are per cent of total radioactivity contributed by unchanged nafcillin as determined by bioassay. Each value is the average from six dogs (see Figs. 1 and 2).

within 6 hours of an intramuscular dose of C^{14}-nafcillin. Bioassay of this urine indicated that only half of the C^{14} present was contributed by microbiologically active drug. From this, it was inferred that the major area of radioactivity at the R_f of nafcillin (0.82), representing 80% of the radioactivity on the paper chromatogram (Fig. 4), contained, in addition to nafcillin, one or more metabolites accounting for 30% of the C^{14} in urine. Two other metabolites, at R_f 0.62 and R_f 0.51, each accounting for 10% of the C^{14} in urine, were found by bioassay of the paper chromatogram to be microbiologically inactive, while the area at R_f 0.82 showed an amount of microbiological activity consistent with the idea that the C^{14} present was divided between nafcillin and metabolite in the ratio 5:3.

One substance considered as a possible metabolite, 2-ethoxynaphthoic acid, was excluded because of its high R_f value, 0.92, as compared with the highest zone of radioactivity at R_f 0.82. The possibility that 2-ethoxynaphthoic acid was formed, but escaped detection by being further metabolized was considered, but ruled out on two counts: when 2-ethoxynaphthoic acid was administered to dogs both unchanged acid and a metabolite (presumably the naphthuric acid) appeared in the urine in about equal quantities, and therefore, one would expect to find some free ethoxynaphthoic acid present if hydrolysis did occur; secondly, nafcillin is resistant to hydrolysis by cultures of *Alcaligenes faecalis* under conditions in which benzylpenicillin is cleaved.

Attempts to identify metabolite *A* as either the penicilloic or penilloic acid of nafcillin have thus far been inconclusive.

Bioassay/Radioassay Ratios.—Values obtained by bio- and radio-assay in urine and plasma showed a consistently lower ratio (bioassay/radioassay) in

TABLE IV.—EXCRETION OF NAFCILLIN IN BILE AND URINE OF DOGS

Time hr.	Animal No.	Bile: Cumulative % of Dose	Bile: Bioassay, % / Radio-	Urine: Cumulative % of Dose	Urine: Bioassay, % / Radio-	Plasma: Total Activity, mcg./ml.	Plasma: Bioassay, % / Radio-
			Intramuscular[a]				
1/2	1	17.4	72	2.0	84	2.7	8
	2	11.6	109	0.05	...	2.4	20
1	1	30.0	71	3.3	80	2.3	20
	2	32.9	70	1.40	82	3.0	12
2	1	48.1	60	6.3	58	1.6	13
	2	61.6	77	4.9	57	2.2	6
4	1	63.1	32	9.8	50	1.3	10
	2	75.3	100	10.6	42	2.0	0
6	1	70.2	37	11.6	33	1.2	0
	2	79.8	50	12.3	55	1.6	0
24	1	79.8	...	13.6	0	1.1	0
	2	82.6	5	...	...	1.4	0
			Oral[a]				
1/2	3	0.11	80	0	...	0.2	0
	4	1.43	81	0.03	...	0.4	40
1	3	3.02	48	0.20	0	0.4	0
	4	5.24	83	0.12	...	1.1	10
2	3	5.80	34	1.14	4	0.4	0
	4	11.91	52	1.48	25	1.3	5
4	3	6.83	16	2.63	0	0.3	0
	4	19.18	46	3.68	10	1.5	0
6	3	7.52	3.6	3.72	0	0.2	...
	4	22.74	41	5.19	6	1.1	0
24	3	10.80	0	8.20	0	0.7	0
	4	42.24	14	...	...	1.1	0

[a] Dose: 10 mg./Kg.

plasma (Table III) than in urine. A ratio of one-half to two-thirds was maintained in the urine for the first 6 hours after intramuscular injection while the drop in plasma bioassay accompanied a change in the ratio from one-third to zero.

When bile and urine were collected after intramuscular administration of nafcillin in a separate experiment, bile and urine maintained fairly high bioassay/radioassay ratios for the first 6 hours (the bioassay ranging from 32 to 100% of the radioassay) but plasma samples bioassayed 20% or less for the first 4 hours and *nil* at the sixth hour. By comparing the amounts of the drug excreted in bile (Table IV) with those finally appearing in feces (Fig. 3), a fair degree of reabsorption of nafcillin from gut is apparent.

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Physics of Tablet Compression XIII

Development of Die-Wall Pressure During Compression of Various Materials

By JOHN J. WINDHEUSER†, JAGDISH MISRA, STUART P. ERIKSEN, and TAKERU HIGUCHI

A method for determining the pressure transmitted to the die wall during tablet compression is described. Basically, the method involves strain-gauge measurement of die expansion and the relation of this to the transmitted pressure. The pressure transmitting behavior of a number of compounds, both organic and inorganic, have been studied; some correlations between observed response and physical properties are suggested. Limited studies on the effect of the addition of a lubricant to the crystals are also reported. It was noted that change in the magnitude of transmission to the die wall was not a simple linear function of lubricant concentration.

EARLIER reports (1–6) of measurements of several variables involved during compression of pharmaceutical tablets did not attempt to determine the relative magnitude of the lateral forces developed during formation of compressed tablets on the die wall by various types of materials. Nelson (7), reporting earlier from these laboratories, proposed and tested a method for possible measurement of these forces but did not study the comparative behavior of different materials. Results of direct measurements carried out on a number of organic and inorganic substances by a relatively simple technique are presented in this communication.

The perpendicular force developed in a die cavity during tablet formation obviously is related to the flow characteristics of the compressed material. If the confined substance acted simply as a hydraulic fluid, the lateral pressure should be essentially equal to the compressional pressure. Compaction of any common granular solid, on the other hand, would lead to development of much lower sideward pressure—the extent of lowering being (in a sense) a measure of ease of lateral distortion or flow of the compressed material. This property would be reflected in the relative ease of ejection of the formed tablet and the moldability of the compacted mass. All this may depend on such

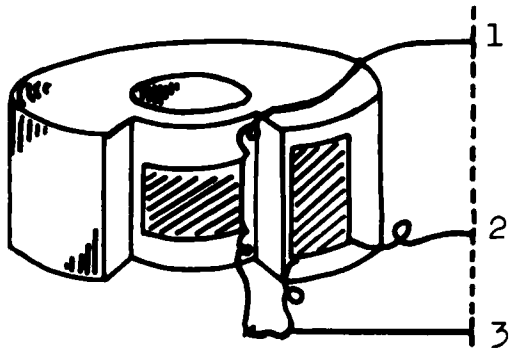

Fig. 1.—Standard steel die modified to accept strain gauges. Numbers refer to connections in circuit diagram in Fig. 2.

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A method for determining the pressure transmitted to the die wall during tablet compression is described. Basically, the method involves strain-gauge measurement of die expansion and the relation of this to the transmitted pressure. The pressure transmitting behavior of a number of compounds, both organic and inorganic, have been studied; some correlations between observed response and physical properties are suggested. Limited studies on the effect of the addition of a lubricant to the crystals are also reported. It was noted that change in the magnitude of transmission to the die wall was not a simple linear function of lubricant concentration.

EARLIER reports (1–6) of measurements of several variables involved during compression of pharmaceutical tablets did not attempt to determine the relative magnitude of the lateral forces developed during formation of compressed tablets on the die wall by various types of materials. Nelson (7), reporting earlier from these laboratories, proposed and tested a method for possible measurement of these forces but did not study the comparative behavior of different materials. Results of direct measurements carried out on a number of organic and inorganic substances by a relatively simple technique are presented in this communication.

The perpendicular force developed in a die cavity during tablet formation obviously is related to the flow characteristics of the compressed material. If the confined substance acted simply as a hydraulic fluid, the lateral pressure should be essentially equal to the compressional pressure. Compaction of any common granular solid, on the other hand, would lead to development of much lower sideward pressure—the extent of lowering being (in a sense) a measure of ease of lateral distortion or flow of the compressed material. This property would be reflected in the relative ease of ejection of the formed tablet and the moldability of the compacted mass. All this may depend on such

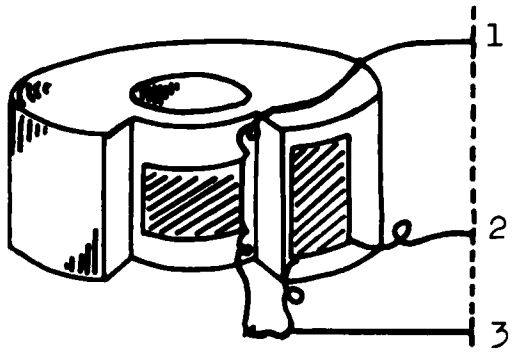

Fig. 1.—Standard steel die modified to accept strain gauges. Numbers refer to connections in circuit diagram in Fig. 2.

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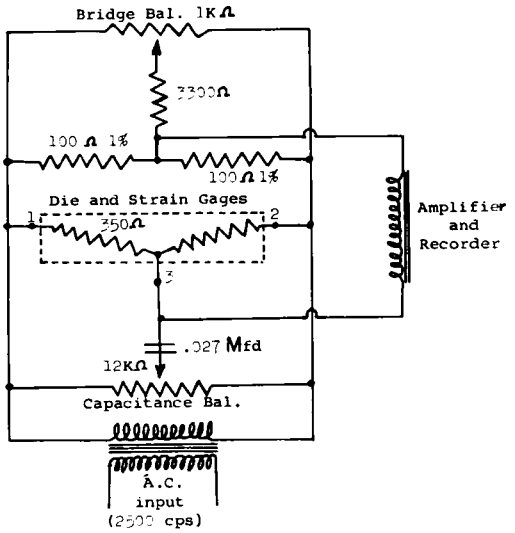

Fig. 2.—Wheatstone bridge arrangement of strain gauges for measurement of die response.

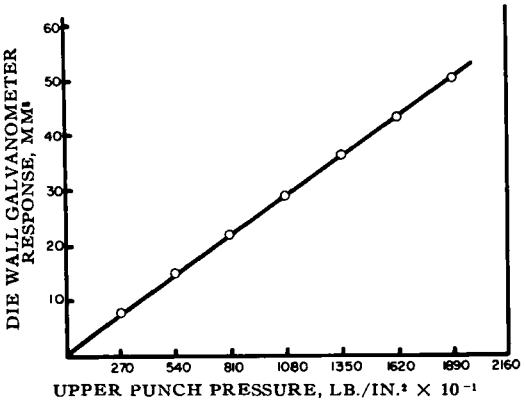

Fig. 3.—Typical calibration curve obtained for die-wall response as a function of applied pressure when using a rubber plug as the transmitting media.

factors as the hardness, shape, and/or the frictional properties of the crystals involved.

Data are presented for a number of halide salts, certain antacids, and a number of organic compounds. Of particular interest are results obtained on phenacetin, acetanilide, and stearic acids—all substances having leaf-like crystalline structure.

EXPERIMENTAL

Instrumentation.—The apparatus used for compression of the tablets was a high force hydraulic press as previously described by Nelson (7). The force exerted by the press was measured with an electrical load cell. In these studies the force applied was measured on the moving upper punch operating against a fixed lower punch. The electrical response of the transducer was followed by a strain-gauge resistance bridge and recorded on one channel of a Sandborn dual-track magnetic oscillograph in the manner previously described (8). The force transmitted to the die wall was recorded simultaneously on the second channel of the recorder as described below. Punches used were $^3/_8$-inch flat faced; the die was adapted from a standard steel $^3/_8$-inch die for the Stokes model A-3 tablet machine.

The die was modified as shown in Fig. 1. A segment of the wall was ground out to increase the sensitivity of the system and to render the stress forces more parallel in the sensed area. Two strain gauges were mounted on the die wall, one normal and the other parallel to the die bore. The strain gauges formed a part of a Wheatstone bridge circuit as shown in Fig. 2. The gauge placed parallel to the die bore was introduced into the circuit to compensate for temperature changes during the compression cycle. The response of the strain gauges was measured and recorded on the Sanborn magnetic oscillograph as noted earlier for the upper punch response. This system offered the advantages of not altering the internal die bore and not having the measurements depend on extrusion characteristics (which have been shown to vary widely for different substances) (9).

Instrument Calibration.—The upper punch response was calibrated by applying known forces

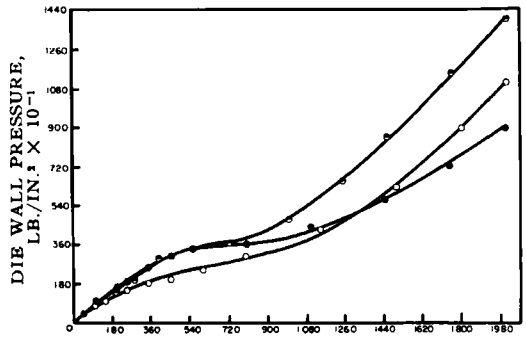

Fig. 4.—Die wall pressure response curve as a function of applied punch pressure for the chloride salts of lithium, sodium, and potassium. ●, LiCl; ○, NaCl; ◒, KCl.

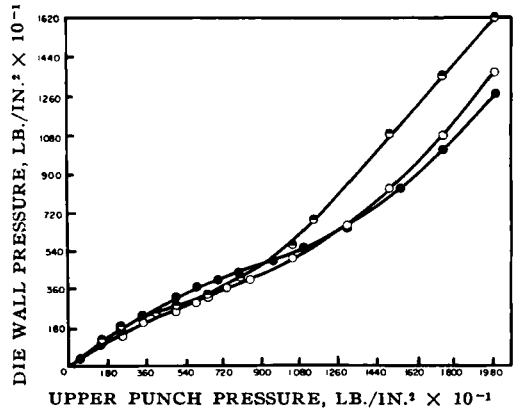

Fig. 5.—Die wall pressure response curve as a function of applied punch pressure for the bromide salts of lithium, sodium, and potassium. ●, LiBr; ○, NaBr; ◒, KBr.

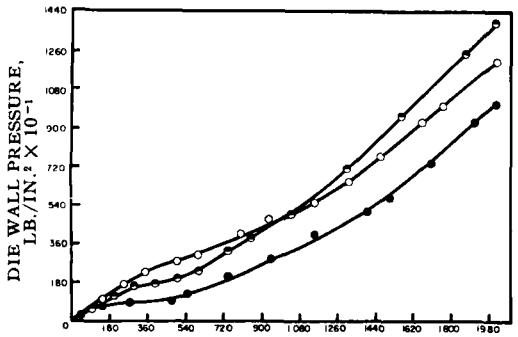

Fig. 6.—Die wall pressure response curve as a function of applied punch pressure for the iodide salts of lithium, sodium, and potassium. ●, LiI; ○, NaI; ⊖ KI.

with a lever arm, the applied force-response curve being essentially linear over the force range employed. The exact response of the die wall to known lateral pressure was determined by inserting a rubber plug into the die bore and measuring the response of the strain gauges to varying forces applied to the upper punch.

According to Adams and Gibson (10), rubber behaves under compression essentially as a liquid, so it was chosen for the calibration. Because of this apparent hydraulic behavior of rubber, the pressure applied by the upper punch can be assumed to be same as that transmitted directly to the die wall. Figure 3 shows a typical calibration curve for the electrical die wall response as a function of applied pressure. These determinations were repeated throughout the study to compensate for strain gauge drift. The response of the die wall strain gauges to a given normal compressional force obviously is a function of the rubber plug or tablet thickness. To eliminate this variable, a constant final thickness of 5 mm. was adopted for all experiments. Although some volume contraction occurs during compression, it was found that this change was not sufficient to alter seriously the results observed for rubber. In the case of tablets, only a 5% reduction in height was noted from the point of initial pressure response to the final thickness of 5 mm. This change appeared to be negligible based on experiments with tablets of varying final thickness.

Compression.—Compression of the material under test was carried out in a manner generally similar to that described previously (7) but with certain modifications. Since the observed response was dependent on final tablet thickness, preliminary trials were necessary for each compound to determine the weight which would yield a 5-mm. tablet. Also all dies were surface lubricated prior to use. In preliminary investigations apparent increases in die-wall forces were observed when unlubricated material was compressed in an unlubricated die as compared to a die with walls coated with a hydrocarbon lubricant.[1] It was felt that the increased response was a function of particle–die wall friction and may be due to the torque exerted on the die by the granulation during compression. As the purpose of the research was to study the effects of particle-particle interaction on the transmission of the forces to the die wall, all investigations were conducted using a die which was lubricated prior to each compression.

Compounds Tested.—All substances tested were of highest chemical purity commonly obtainable. They were dried at suitable temperatures and stored in vacuum dessicators prior to use. Storage was found necessary since variation in moisture content appeared to alter the compression characteristics.

RESULTS AND DISCUSSION

Effect of Particle Size

Changes in the particle size of the material subjected to compression appear to have only a minor (if any) effect on the side wall pressure transmission. A sample of sodium chloride crystals was fractionated by using standard screens in three general size ranges: larger than 20-mesh, through a 20-mesh and caught on a 40-mesh screen, through a 40-mesh and retained on a 60-mesh screen. Within experimental variation, the curves were identical with that for an unsegregated sample indicating no significant difference arising from particle-size variation over the range studied. For this reason no serious effort was made in the succeeding determinations to control particle sizes.

Compression of Inorganic Compounds

Alkali Metal Halides.—The compressional characteristics of the chloride, bromide, and iodide salts of lithium, sodium, and potassium were studied in some detail since these compounds were available in pure states and because of the availability of precise effective radii of the ionic components. Since the internal crystal attractions (and consequently crystal hardness) appear to depend on inter-ionic distances, some correlation was expected between these values and the efficiency of pressure transmission.

Figures 4–6 show the typical relationships found between applied pressure and the pressure transmitted to the die wall during several runs carried out on the halide salts. In all cases the general shapes of the curves were similar, they had an initial linear segment, then passed through an area having reduced transmittance characteristics, and then a subsequent linear portion which in most cases appeared to approach a slope of one. Although duplicate runs on a given material showed some variation, these variations were small relative to the dif-

TABLE I.—INITIAL SLOPE $\left(\frac{\Delta dw}{\Delta up}\right)$

	Cl^-	Br^-	I^-
Li^+	0.80	0.70	0.54
Na^+	0.75	0.60	0.65
K^+	0.71	0.70	0.56

TABLE II.—APPARENT YIELD VALUE[a] IN LB./IN.²

	Cl^-	Br^-	I^-
Li^+	>12,000	9,000	9,100
Na^+	9,100	8,600	7,500
K^+	6,300	5,400	5,700

[1] Lubriseal—Arthur H. Thomas Co., Philadelphia, Pa.

[a] Final slope extrapolated to zero-die response.

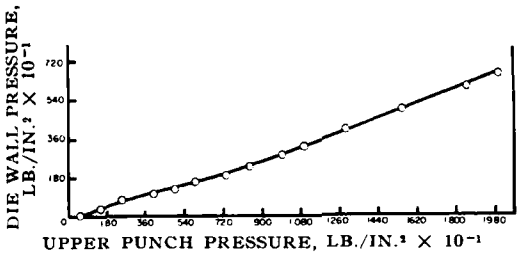

Fig. 7.—Die wall pressure response curve of DASC as a function of applied upper punch pressure.

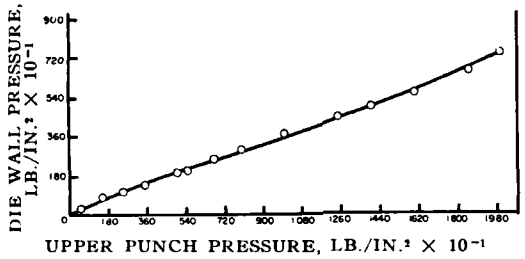

Fig. 8.—Die wall pressure response curve of potassium carbonate as a function of applied upper punch pressure.

ferences observed between materials. Usually replicate sidewall pressure determinations agreed within ±5% for a given substance at a particular compressional pressure.

Although the present state of knowledge cannot totally explain the observed results, some rationalization is possible. For example, the die-punch response curve may depend on crystal hardness, particle-particle friction, and/or shape of the crystal. Table I shows that the initial slopes of the curves plotted in Figs. 4–6 appear to follow the pattern of the hardest crystals (LiCl) having the highest initial slopes, and the softest (KI) having the lowest initial slopes, although exceptions appear to exist. The slopes seem to be somewhat more dependent on the nature of the anionic than on the cationic part.

The subsequent decrease in slopes occur in the compressional range associated with the fracturing of crystals and the assumption of increasingly closer packing. That the fraction of the pressure transmitted to die wall is reduced in this region is certainly not unexpected. The final slope, approaching a direct transmission condition, may represent a void-free, solid-state compression and flow. Table II represents a tabulation of the apparent "yield values" for the salts investigated. These values, which are the extrapolations of the final slopes back to zero die wall response, may be expected to increase with crystal hardness if the previous assumptions have some validity. This seems to be generally the case.

Some Antacids.—Figures 7 and 8 show the data obtained when dihydroxy aluminum sodium carbonate[2] and potassium carbonate were compressed. Both exhibited a rather low degree of transmission of the punch force to the die wall, indicating relatively poor flow tendency. During the compression and ejection of the tablets, the machine emitted sounds which indicated marked binding between tablet and die wall. The low initial slope shown by the DASC curve was also reflected by stratification within the finished tablet (inidicative of layer formation).

[2] DASC-Chatanooga Chemical Brand of dihydroxy aluminum sodium carbonate.

Compression of Organic Substances

Lateral pressure transmission characteristics of some organic compounds of pharmaceutical importance were studied. The results of these investigations are shown in Figs. 9 and 10. Although the shapes of the curves are similar to those observed with the alkali metal halides, the curves for benzocaine, aspirin, and phenobarbital do not exhibit the pronounced leveling that was previously found. This might be attributed to the crushing of the crystals even at low pressure and the assumption of a state of closest packing, followed by essentially void-free compression. In the case of the carbohydrates, the plateau is more extended as might be expected from the relative hardness of the sugars (in comparison to the other compounds tested). From Figs. 9 and 10 the "yield values" of aspirin and lactose were found to be 5800 lb./in.² and 12,700 lb./in.², respectively. Higuchi, *et al.* (3), had found earlier that lactose appeared to be three to four times as hard as aspirin. This investigation agreed qualitatively with the apparent "yield values" found in this study.

It is a well known fact that certain compounds are relatively difficult to compress into acceptable tablets. Phenacetin is an outstanding example of a

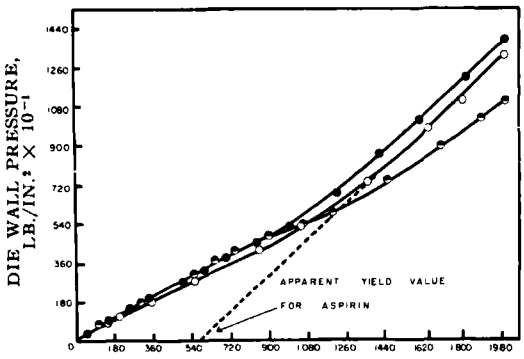

Fig. 9.—Die wall pressure response curve of benzocaine, aspirin, and phenobarbital as a function of applied upper punch pressure. ●, Benzocaine; ○, aspirin; ◒, phenobarbital.

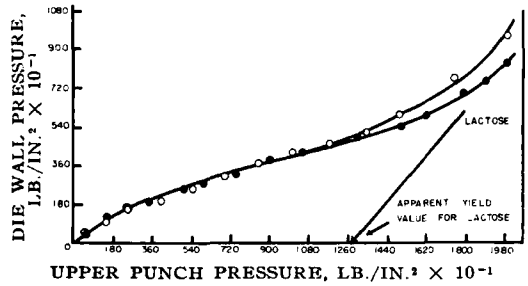

Fig. 10.—Die wall pressure response curve of sucrose and lactose as a function of applied upper punch pressure. ○, Sucrose; ●, lactose.

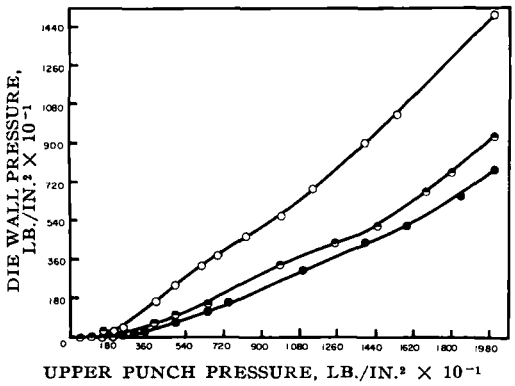

Fig. 11.—Die wall pressure response curve of stearic acid, acetanilid, and phenacetin as a function of applied upper punch pressure. O, Stearic acid, m.p. 69–70°; ◓, acetanilid, m.p. 113–115°; ●, phenacetin, m.p. 134–135°.

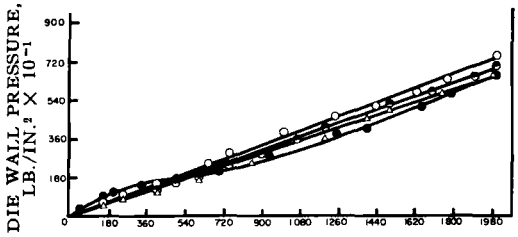

Fig. 12.—The effect of lubrication with magnesium stearate on the die wall–punch pressure response curve of sulfathiazole. Key: ●, unlubricated sulfathiazole; O, sulfathiazole plus 0.25% magnesium stearate; ◓, sulfathiazole plus 0.50% magnesium stearate; Δ, sulfathiazole plus 1.00% magnesium stearate.

substance yielding tablets which are prone to capping. Such tablets when cleaved show indications of lamination. It was felt that this behavior may be reflected in the die wall–punch response curve.

Figure 11 shows the results obtained during compression of crystalline stearic acid, acetanilid, and phenacetin. The essentially zero initial slope in the plots is common to all three compounds, indicating almost no lateral force transmission during the initial phase of compression. After this stage the shape of the curve is similar to that observed with the alkali halides, except that the plateau region is less pronounced than that previously observed.

This behavior may be related to the shape factor mentioned earlier. Each of these three compounds exists in plate-like crystals which might tend to form layers under compression. This layering effect would reduce the lateral forces observed and possibly contribute to the tendency to cap. Although "yield values" were not determined since the final slopes did not appear linear at the higher pressures, it is apparent that qualitatively they would be in the expected order of the softest crystal—stearic acid having the lowest value and the hardest phenacetin having the highest.

Effect of Lubricants

In the previous work by Nelson (7), it appeared that the addition of to 1.6% magnesium stearate to a sulfathiazole granulation increased the force transmitted to the die wall by approximately 25%. Observations made as a part of this study with sulfathiazole and potassium chloride crystals lubricated by addition of 0.25, 0.5, and 1% magnesium stearate differed to some extent. The lubricant was incorporated into the system by trituration. The results are depicted in Figs. 12 and 13. In sulfathiazole, the unlubricated material exhibited the typical sigmoid curve but with lower transmittance than other organic compounds tested.

The lubricated samples, contrary to expectations, showed lower transmission characteristics at low punch pressures than the unlubricated materials, but higher ratios of the die-wall response at high pressures. It was surprising that transmission decreased with an increased addition of lubricant after an initial rise. A maximum increase of 10% transmission was found at higher pressures when 0.25% magnesium stearate was added, but only a 5–7% increase was recorded at 1% lubricant concentration. Similar results were found in the compression of potassium chloride, except that the increase with 0.25% magnesium stearate was more pronounced

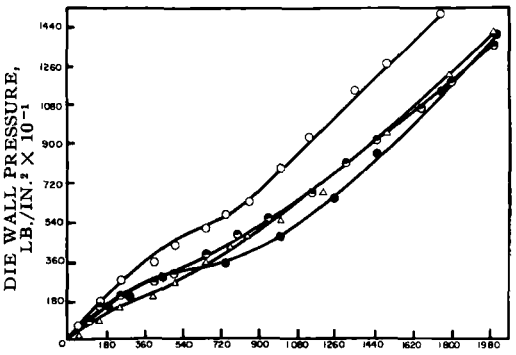

Fig. 13.—The effect of lubrication with magnesium stearate on the die wall–punch pressure response curve of potassium chloride. ●, Unlubricated KCl; O, KCl plus 0.25% magnesium stearate; ◓, KCl plus 0.50% magnesium stearate; Δ, KCl plus 1.00% magnesium stearate.

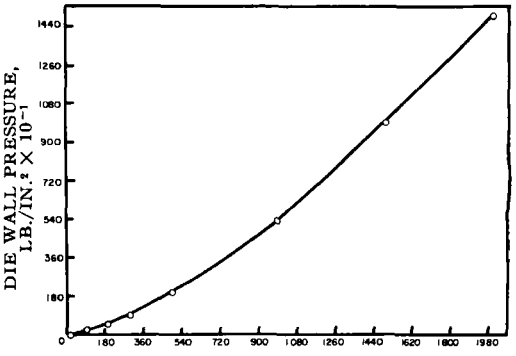

Fig. 14.—Die wall pressure response curve of magnesium stearate as a function of applied upper punch pressure. O, Magnesium stearate.

than with sulfathiazole; however, it presented the same trend at higher lubricant concentration that had been found with the sulfonamide.

The reason for the observed behavior may be found in the compressional characteristics of magnesium stearate. Figure 14 shows the curve obtained for the compression of pure magnesium stearate which is qualitatively similar to the curve shown for stearic acid in Fig. 11. The poor transmission behavior under lower pressure might give rise to the effect seen above, but this is not clearly established.

CONCLUSION

Although these studies have been largely preliminary, the results suggest that measurements of lateral pressure developed during formation of pharmaceutical tablets may provide a useful indication of compressional characteristics of various materials. As a broad and possibly too sweeping conclusion, it appears that materials which permit rather good conversion of normal pressure to lateral pressure tend to form good tablets. Substances expected to exhibit poor flow properties under pressure, such as those composed of thin, flat, leaf-like crystals, appear to be shown by this technique to behave in this manner.

The general method of study seems to be adaptable to pilot plant and commercial tablet machines with appropriate modifications. It is relatively simple and rapid. The rubber plug technique seems to provide a ready and quick means of calibration.

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Sabadilla Alkaloids VIII

Isolation of Sabadillines I, II, and III

By GLENN R. SVOBODA†, HYMAN MITCHNER‡, and LLOYD M. PARKS§

The techniques of partition and adsorption chromatography as well as countercurrent distribution have been applied to alkaloidal concentrates from both sabadilla and veratrine. Whereas sabadilline previously was considered to consist of a single alkaloidal constituent, the present work has resulted in the isolation of three different materials which possess the characteristic ultraviolet absorption maximum at 238 mμ. These materials have been named sabadilline I, II, and III. Only sabadilline II was obtained in crystalline form. Attempts to establish a relationship between the three compounds were unsuccessful. Only sabadilline II yielded an alkaline isomerization product and this was not similar to either sabadillines I or III.

THE PRESENCE of an alkaloidal constituent of *Schoenocaulen officinale* (sabadilla) which exhibited an ultraviolet maximum at 238 mμ was first noted by Poetsch (1). This material was obtained from a commercial concentrate sold under the name "sabadilline." This was the name applied to the crystalline material, isolated by Hennig (2), which exhibited a similar ultraviolet spectrum. The work of Stuart (3) and Mitchner (4) confirmed the presence of a sabadilline-like material in both sabadilla and the commercial concentrate, veratrine, an alkaloidal extract of sabadilla.

A material which appeared to be similar to sabadilline was isolated by Auterhoff (5) and partially characterized by Vejdelek, Macek, and Kakac (6). To this material, which was named veragenine, was attributed an $\alpha\beta$-unsaturated ketone structure, unknown for any isolated sabadilla constituent other than sabadilline, for which an $\alpha\beta$-unsaturated ketone structure previously had been postulated by Stuart (3). The possibility of a similarity between the two compounds indicated the necessity of further investigation of the substance which exhibited

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The authors are indebted to Dr. K. K. Chen and Dr. R. G. Herrman of Eli Lilly and Co. for the cited pharmacological results.
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The authors are indebted to Dr. K. K. Chen and Dr. R. G. Herrman of Eli Lilly and Co. for the cited pharmacological results.
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the ultraviolet maximum at 238 mμ first noticed by Poetsch (1). Before this could be done it was necessary to devise a more convenient separation procedure than the chromatographic separation devised by Hennig (2).

EXPERIMENTAL

Materials.—All chemicals except technical chloroform were of reagent quality. Ultraviolet spectra were obtained with a Cary model 11S automatic recording spectrophotometer. Infrared spectra were obtained with a Baird double-beam instrument. Buffers were standardized with a Beckman model H-2 pH meter. The Craig countercurrent distribution apparatus used was a 200 tube, robot driven instrument (manufactured by H. O. Post, Maspeth, N. Y.). Each tube was of 20-ml. capacity, adjusted for 10 ml. upper and lower phase. A 2-ml. microburet was used for the titrations. Samples were titrated in chloroform *versus* perchloric acid in glacial acetic acid using quinaldine red as the indicator. The veratrine used in this investigation was kindly supplied by S. B. Penick and Co.

Purification of Sabadilline Concentrates.—Available from the isolation of sabatine from Poetsch's Fraction D (1) by partition chromatography of successive samples on silicic acid at pH 8.00, were the peak materials eluted immediately prior to the sabatine peak. These corresponded to the material

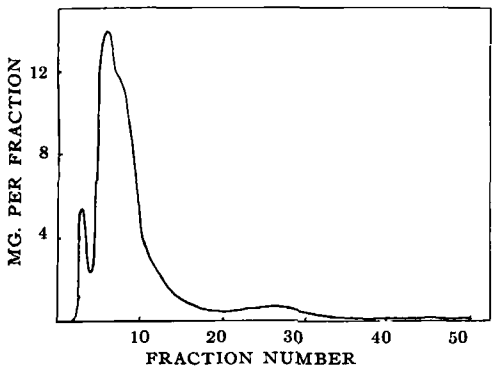

Fig. 1.—Partition chromatogram of a sabadilline concentrate on silicic acid with chloroform *vs.* pH 8.00 phosphate buffer.

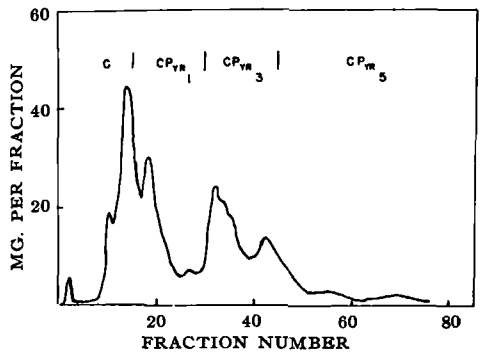

Fig. 2.—Partition chromatogram of a sabadilline concentrate on purified cellulose with chloroform and chloroform containing the designated increments (%) of pyridine *vs.* pH 6.50 phosphate buffer.

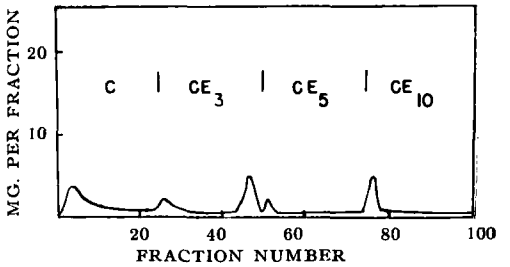

Fig. 3.—Adsorption chromatogram of a sabadilline concentrate on activated (basic) alumina with chloroform and chloroform containing the designated increments (%) of ethanol.

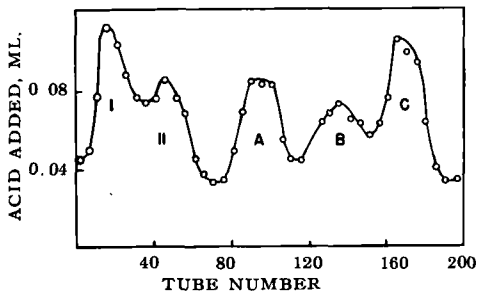

Fig. 4.—Countercurrent distribution of 1 Gm. of Fraction I at pH 6.35 for 406 transfers *vs.* chloroform.

from which Hennig (2) was able to isolate a few milligrams of sabadilline. Ultraviolet absorption analyses of these peak materials confirmed the presence of a maximum at 238 mμ. These sabadilline concentrates were purified by a modified countercurrent distribution using a chloroform–phosphate buffer (pH 7.25) system.

The sabadilline concentrates were dissolved in 50 ml. of chloroform and shaken with an equal volume of pH 7.25 phosphate buffer. The chloroform solution was transferred to the next separator containing fresh buffer at pH 7.25. The process was continued through six transfers. The buffer solutions were combined, made alkaline with ammonium hydroxide, and extracted with 10-ml. portions of chloroform until a negative Mayer's test was obtained. The chloroform extracts were filtered through anhydrous sodium sulfate and evaporated to dryness under reduced pressure. Successive peak materials were treated in a similar manner. Those materials which exhibited an ultraviolet maximum at 238 mμ were combined and used in chromatographic separations.

Partition Chromatography with Sabadilline Concentrates.—Silicic acid columns (30 Gm. silicic acid:30 ml. buffer) were prepared as described by Poetsch (1). A 15-to-1 ratio of length-to-width was maintained for all columns. Samples (about 500 mg.) were dissolved in a minimal volume of chloroform and placed on the column. Ultraviolet analyses of 10-ml. fractions showed that the sabadilline-like material was eluted from the negative slope of the main elution peak. Alkaloidal fractions which exhibited the ultraviolet absorption maximum at 238 mμ from several such columns were combined (125.6 mg.) and placed on a column which consisted of 6 Gm. of silicic acid and 6 ml. of pH 8.00 phosphate buffer. Ultraviolet analysis of the chloroform elu-

tion pattern obtained (Fig. 1) indicated the possibility of two independent materials which exhibited a sabadilline-like absorption spectrum based upon the extinction coefficients obtained.

Another supporting phase used for the partition chromatographic separation of sabadilline concentrates was Solka-Floc BW-200, a purified cellulose (obtained from the Brown Co., Boston, Mass.). A 1.0-Gm. sample of sabadilline concentrate was placed on a column which consisted of 40 Gm. of cellulose and 30 ml. of pH 6.50 phosphate buffer. Figure 2 shows the elution pattern of 10-ml. fractions obtained using chloroform and chloroform-containing increments of pyridine.

Ultraviolet analyses of the eluted fractions indicated the existence of three independent materials which exhibited the sabadilline maximum at 238 mμ. Fractions 13 to 20 were combined to give about 300 mg. of material which had an extinction coefficient ($k \times 100$ at 238 mμ) in excess of 1000. All attempts to crystallize this material were unsuccessful.

Adsorption Chromatography with Sabadilline Concentrates.—The adsorbents investigated in this study included silicic acid, acid-washed alumina, activated alumina, light and heavy magnesium oxides, Florisil, and Celite. Of these, only activated alumina gave results worth further consideration. Ultraviolet absorption analyses of the 10-ml. fractions obtained by the chromatographic separation of a 100-mg. sample of a sabadilline concentrate on 5.0 Gm. of activated alumina using chloroform and chloroform-containing increments of ethanol (Fig. 3) indicated the existence of three sabadilline materials.

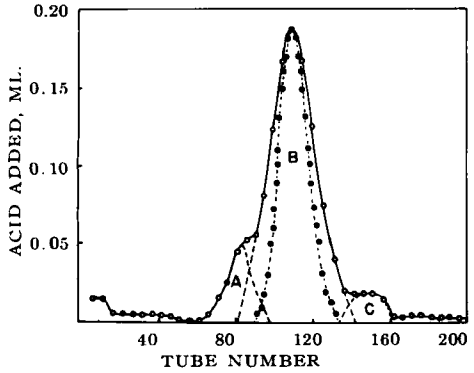

Fig. 5.—Countercurrent distribution of Peak B, Fraction I at pH 6.35 for 400 transfers *vs.* chloroform. Key: O, experimental; ⊖, theoretical.

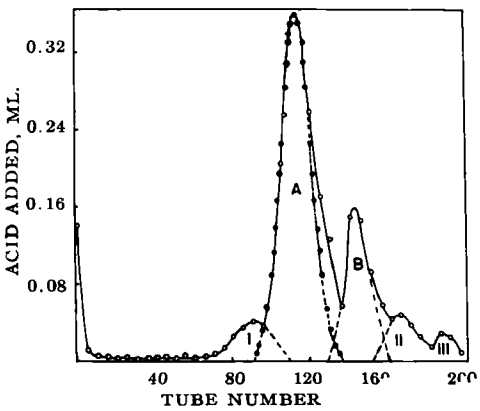

Fig. 6.—Countercurrent distribution of 1.0 Gm. Fraction III at pH 6.65 for 210 transfers *vs.* chloroform. Key: O, experimental; ⊖, theoretical.

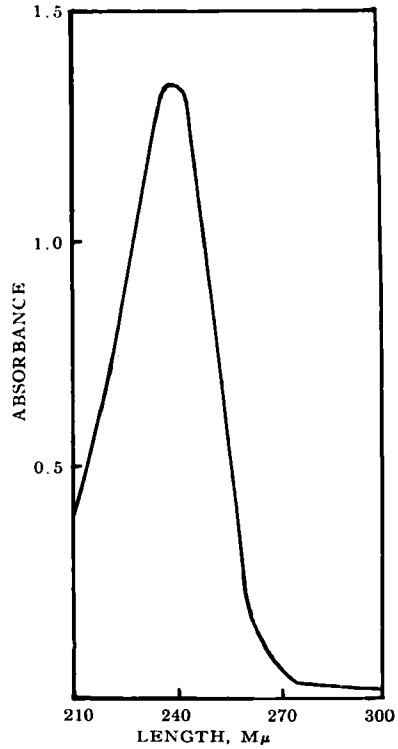

Fig. 7.—Ultraviolet spectrum of sabadilline II in absolute ethanol ($k \times 100 = 3004$).

The other adsorbents cited gave either too large a sample holdup or a lack of constituent resolution.

At this point it was deemed advisable to investigate other means of separation.

Countercurrent Distribution of Sabadillines Obtained from Veratrine.—Previous work (7) describing the quantitative determination of the known alkaloidal constituents of commercial veratrine described the isolation of the hydrophilic alkaloids in $P_{0.1.2}$. Successive 5-Gm. samples of $P_{0.1.2}$ were distributed for about 200 transfers at pH 7.00 *versus* chloroform. Five distribution peaks were obtained. Ultraviolet absorption analyses of the negative slopes of (*a*) Peak A, (*b*) Peak C, and (*c*) the positive slope of Peak E, indicated the presence of a maximum at 238 mμ. Alkaloidal material which was interposed between (*a*), (*b*), and (*c*) did not exhibit ultraviolet maxima at 238 mμ. Infrared absorption analyses confirmed the presence of an unsaturated band for (*a*), (*b*), and (*c*) and the absence of this band for the interposed material. The tubes which corresponded to these areas of the distributions were combined to give Fractions I to VI.

Successive portions of Fractions I, III, and V were subjected to countercurrent distribution until nearly identical theoretical and experimental curves were obtained or until the sample size prevented further purification; then attempts were made to crystallize the material. Distribution patterns were obtained as described previously (7).

Six portions of Fraction I (100 to 1100 mg.) were

distributed at pH values from 5.50 to 6.35 for 100 to 400 transfers. The best resolution of material was obtained with a 1.0-Gm. sample at pH 6.35 *versus* chloroform for 400 transfers. All alkaloidal material was found as shown in the first 200 tubes of Fig. 4. Peak B was the only material which exhibited an ultraviolet maximum at 238 mμ. All of the material of this peak was again distributed at pH 6.35 *versus* chloroform for 400 transfers. All of the alkaloidal material was found as shown in the first 200 tubes of Fig. 5. Although the distribution was symmetrical, there was no agreement between theoretical and experimental points. All attempts to crystallize the material obtained from Peak B, Fraction I were unsuccessful.

Amorphous sabadilline I (Peak B, Fraction I) softened from 126–133°, melted at 133–137°, and remelted from 133–140°. The material decomposed above 250°. Elemental analyses were inconclusive. The infrared spectrum of sabadilline I is shown in Fig. 8a. The alkaloidal spectrum (A.S.) value of sabadilline I was 6.05. The designation of alkaloidal constituents by A.S. value is helpful for unknown constituents. These constants are represented by that pH at which the partition coefficient of the constituent in chloroform *versus* aqueous buffer is unity (4). The A.S. values allow the accurate characterization of new components with respect to their A.S. values and give partition coefficient data for each unknown.

Sabadilline II was obtained from Fraction III in the following manner. Two samples of Fraction III (1.0 and 0.8 Gm.) were individually distributed for 210 transfers at pH 6.65 *versus* chloroform. The distribution pattern of the 1.0-Gm. sample is shown in Fig. 6. The distribution pattern was similar for the 0.8-Gm. sample. The materials found in Peaks A and B both exhibited an ultraviolet maximum at 238 mμ. The material in Peak A had an A.S. value of 6.72. However, the A.S. value of the material in Peak B (6.98) corresponded to the A.S. value of the material obtained by the aqueous alkaline treatment of Fraction III (*vide infra*). Therefore, Peak A was

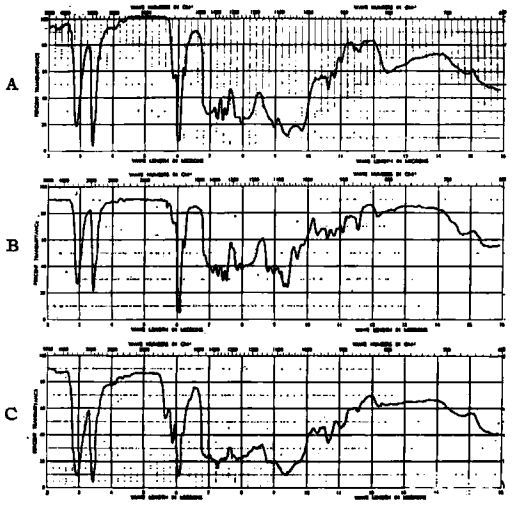

Fig. 8.—(A) Infrared spectrum of amorphous sabadilline I. (B) Infrared spectrum of crystalline sabadilline II. (C) Infrared spectrum of amorphous sabadilline III.

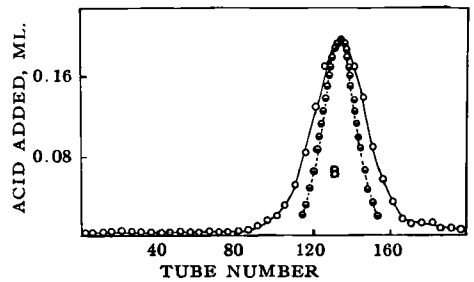

Fig. 9.—Countercurrent distribution of Peak B, Fraction V at pH 7.80 for 214 transfers *vs.* chloroform. Key: ○, experimental; ◒, theoretical.

considered to consist of naturally occurring sabadilline II. Crystalline sabadilline II was obtained from the combined Peak A materials of the two distributions. A total of 100 mg. of water-white, needle-shaped prisms was obtained by crystallization in ethanol-water. The crystals were dried at 100° (0.05 mm.) and exhibited the following melting characteristics: swelled and became opaque from 141–146°, decomposed slightly from 210–225° and melted from 235–252°. The ultraviolet spectrum of sabadilline II is shown in Fig. 7 and its infrared spectrum in Fig. 8b. Elemental analyses were inconclusive.

Sabadilline III was obtained from Fraction V. Five portions of Fraction V of from 100-1000 mg. were distributed at pH values of 7.90 to 8.00 *versus* chloroform for 200 transfers. Only Peak B of Fraction V exhibited an ultraviolet maximum at 238 mμ. The material which comprised Peak B from all of the distributions was combined and distributed for 214 transfers at pH 7.81 *versus* chloroform (Fig. 9). Sabadilline III had a calculated A.S. value of 7.83. There was no agreement between the theoretical and experimental distribution values. Attempts to crystallize sabadilline III were unsuccessful. Amorphous sabadilline III exhibited the following melting characteristics: swelled 126–136°, no true melt to 170°, reswelled from 123–140°, melted from 140 180°, and decomposed slowly above 210°. The infrared spectrum of sabadilline III is shown in Fig. 8c.

Attempts to Determine a Relationship Between Sabadillines I, II, and III.—Countercurrent distribution of the products of methanolysis (8) of Fraction III showed that the reaction conditions were too mild since only the original material appeared in the distribution pattern of the treated material. A similar analysis of the products of ethanolic potassium hydroxide treatment (8) of Fraction III indicated by their number that these reaction conditions were too severe. The conditions finally selected for the methanolic alkaline treatment of Peak B, Fraction III were as follows: A 59-mg. sample of Peak B, Fraction III was dissolved in a solution of 4 ml. of methanol and 1 ml. of 1 *N* potassium hydroxide. The solution was refluxed for 15 minutes, cooled, and made just acid with 1:1 hydrochloric acid. The methanol was removed under reduced pressure and the residue was extracted with four 25-ml. portions of chloroform after adding sufficient ammonium hydroxide to give a pH of 9.00. The combined chloroform extracts were dried by filtration through anhydrous sodium sulfate and evaporated to dryness under reduced pressure. The prod-

uct obtained was distributed for 106 transfers at a pH of 7.00 *versus* chloroform. The single distribution peak obtained was collected and distributed again at pH 7.50 *versus* chloroform. The single peak obtained from the latter distribution had a calculated A.S. value of 6.98 compared to 6.72 for sabadilline II. The material was considered to be an alkaline isomerization product of sabadilline II and was similar to the material obtained from Peak B, Fraction III which had a calculated A.S. value of 6.97.

Peak B, Fraction I and Peak B, Fraction III (sabadilline I and III) were treated in a similar manner. Infrared analysis of the materials obtained in the respective distribution peaks of the reaction products showed that they consisted of impure starting materials. Therefore, the results of the aqueous alkaline treatment of sabadillines I and III were inconclusive, in that they were not changed under the reaction conditions to which they were subjected. The small amount of material available precluded further investigation.

Pharmacological Evaluation of Sabadilline II.— In the dog under phenobarbital anesthesia, 1.0 mg./Kg. intravenously depressed respiration for approximately 10 minutes, depressed gut motility, and produced a slight transitory rise in blood pressure. The EKG record showed depression of the S-T segment with an increase in T-wave amplitude similar to that observed with increased blood potassium level. At 2.19 mg./Kg. intravenously it produced a fall in blood pressure for approximately 5 minutes and a temporary inhibition of respiration and gut motility. After 20 minutes, the heart rate slowed and the dog died. EKG changes were the same as with the 1-mg./Kg. dose.

Because of the amorphous nature of sabadilline I and III, their physiological activity as potential hypotensives was not checked.

DISCUSSION AND CONCLUSIONS

A prerequisite to the successful conclusion of this investigation was to devise separation and purification techniques for sabadilline concentrates. Early chromatographic studies were laborious and confusing, indicating the presence of several materials which possessed the sabadilline ultraviolet maximum at 238 mμ. Additionally, the elution of the possible single compound was discontinuous and the apparent presence of separate compounds of this nature was because of a change in elution solvent composition.

Countercurrent distribution analyses of commercial veratrine had shown that between 1.8 and 4.8% of sabadilline material (as unknown X_1) was present in the total alkaloids (7) This work involved the separation of veratrine into hydrophobic, hydrophilic, and intermediate solubility fractions. Further countercurrent distributions of the hydrophilic alkaloids indicated three of the six fractions contained material which exhibited the sabadilline ultraviolet absorption spectrum. Further separation of the sabadilline-like materials in these fractions utilized additional countercurrent separations as described in this work.

The first of these sabadilline-containing concentrates (Fraction I) was shown to consist of at least five different constituents. The first two peaks obtained (Fig. 4) were not homogeneous. The third peak, Peak A, was known to be cevacine. It was Peak B which yielded sabadilline I. Peak C was shown to be another previously unknown Sabadilla constituent and was obtained in crystalline form. Further description of this material will be the subject of a later publication.

Sabadilline II and a compound believed to be its alkaline isomerization product were isolated by the countercurrent distribution of Fraction III (Fig. 5). Peak A corresponded to sabadilline II; Peak B corresponded to a product obtained by the alkaline treatment of sabadilline II. Sabadilline II was the only sabadilline obtained in crystalline form. The infrared spectrum of sabadilline II suggests a structure which more closely resembles sabine (4) than veracevine (6), thus suggesting that the parent alkamine of sabadilline is structurally most like sabine.

The third of the sabadilline compounds was obtained by countercurrent distribution of Fraction V (Fig. 6). Peak B was shown to consist of amorphous sabadilline III.

There was no question of the independent nature of the three sabadillines. It was then of interest to determine if there was some simple relationship between these alkaloids. The fractions from which the sabadillines were isolated were subjected to aqueous alkaline treatment. Infrared spectra before and after such treatment showed no significant structural changes resulting from the conditions of such treatment. Herein is sufficient evidence that the $\alpha\beta$-unsaturated ketone structure attributed to the sabadillines does not arise from the isolation procedures inasmuch as these procedures are far more mild than the aqueous alkaline treatment.

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Sabadilla Alkaloids IX

Isolation of Unreported Minor Constituents and Their Relationship to the Known Sabadilla Alkaloids

By GLENN R. SVOBODA†, HYMAN MITCHNER‡, and LLOYD M. PARKS§

Additional minor alkaloidal constituents of sabadilla have been isolated from veratrine. Four of these were obtained as symmetrical countercurrent distribution peaks. A fifth, sabadene, was obtained in crystalline form. Sabadene possessed no hypotensive properties. The investigation also notes the existence of extremely minor quantities of unresolved constituents. A review of the relationship of all reported sabadilla alkaloids is presented.

As INDICATED in previous reports (1, 2), additional unreported alkaloidal constituents of sabadilla have been isolated by countercurrent distribution of commercial veratrine. One of these, for which the name "sabadene" is proposed, was obtained in crystalline form. Sabadene was shown by infrared analysis to contain an isolated carbon-carbon double bond structure, previously unknown for any sabadilla alkaloid. In addition, four other amorphous alkaloidal materials were obtained as symmetrical distribution peaks and two additional distribution peaks were obviously of a heterogeneous nature.

Fortunately, the isolation procedure resulted in the isolation of previously known sabadilla constituents for which pH assignments have been made at which these constituents demonstrate a partition coefficient of unity in aqueous buffers *versus* chloroform. The constants represented by partition coefficients equal to one have been called alkaloidal spectra (A.S.) values (3). These values allowed the accurate characterization of the new components with respect to their relative A.S. values and gave partition coefficient data for each unknown.

None of the new alkaloidal constituents described herein exhibited a characteristic ultraviolet spectrum. However, vanilloyl veracevine which had been reported previously (4) did exhibit its characteristic ultraviolet spectrum. The description of the original isolation of vanilloyl veracevine did not cite its partition characteristics relative to the total sabadilla constituents as is done in the present work.

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EXPERIMENTAL

Apparatus and Reagents.—These were the same as described previously (2). The isolation techniques and distribution determinations have been similarly described (2).

Constituents of Fraction I.—The distribution

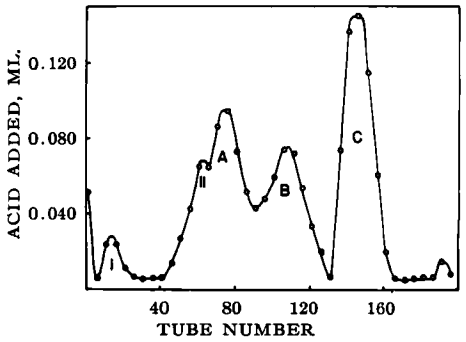

Fig. 1.—Countercurrent distribution of a 1.0-Gm. sample of Fraction I at pH 6.35 for 433 transfers *vs.* chloroform.

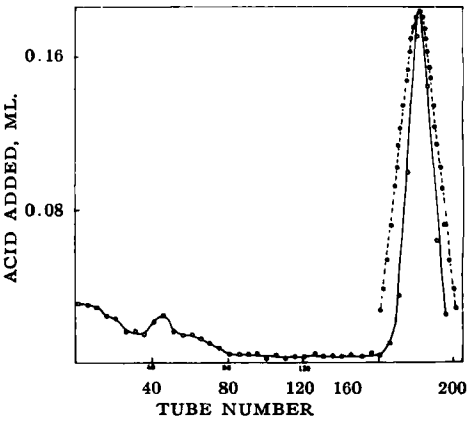

Fig. 2.—Countercurrent distribution of Peak I, Fraction I at pH 5.13 for 427 transfers *vs.* chloroform. Key: ○—experimental, ◒—theoretical.

conditions which resulted in the best resolution of the constituents of consecutive 1.0-Gm. samples of Fraction I were a pH of 6.35 and about 400 transfers *versus* chloroform. Figure 1 shows the results obtained, all of the alkaloidal constituents being present in the first 200 tubes. The alkaloidal material which constituted each of the individual peaks was collected and treated as follows.

Peak I, Fraction I, was collected and distributed at pH 5.13 for 427 transfers *versus* chloroform, all of the alkaloidal constituents being present in the first 200 tubes (Fig. 2). The calculated A.S. value of the single symmetrical peak was 5.00. Peak I, Fraction I, exhibited the following melting characteristics: melted at 123–131° and remelted at 153–158°.

The distribution of Peak II, Fraction I, at pH 5.47 for 403 transfers *versus* chloroform (Fig. 3) showed that the original peak was actually composed of at least three materials as well as a residual quantity of Peak I, Fraction I. In this distribution, all of the alkaloidal material was present in tubes 120 to 280. The identity of residual Peak I, Fraction I, as an unknown was confirmed by the calculated A.S. value (5.25) of Peak I (Fig. 3) compared to the value obtained for Peak I, Fraction I (5.20), of Fig. 2.

Peak A, Fraction I, was distributed at pH 5.88 for 464 transfers *versus* chloroform (Fig. 4). Peak A had a calculated A.S. value of 5.89 compared to 5.88 for cevacine and exhibited an infrared spectrum similar to that of authentic cevacine.

The treatment of Peak B, Fraction I, from which sabadilline I was isolated has been described previously (2).

The fractions which constituted Peak C (Fig. 1) were combined and yielded 159 mg. of crystalline

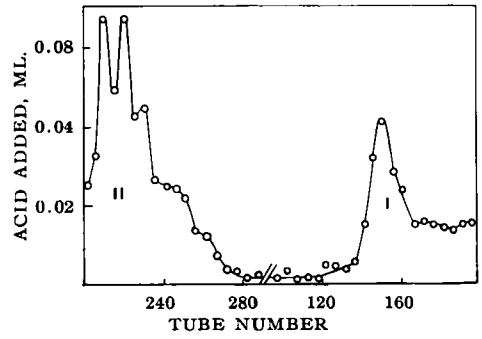

Fig. 3.—Countercurrent distribution of Peak II, Fraction I at pH 5.47 for 403 transfers *vs.* chloroform.

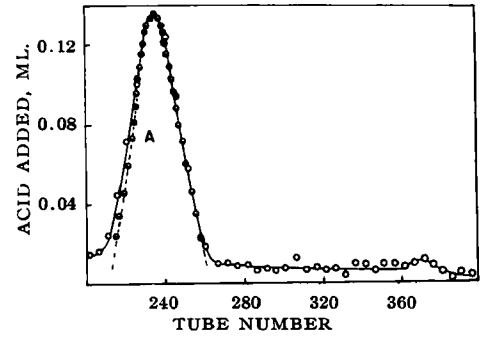

Fig. 4.—Countercurrent distribution of Peak A, Fraction I at pH 5.88 for 464 transfers *vs.* chloroform. Key: ○—experimental, ◒—theoretical.

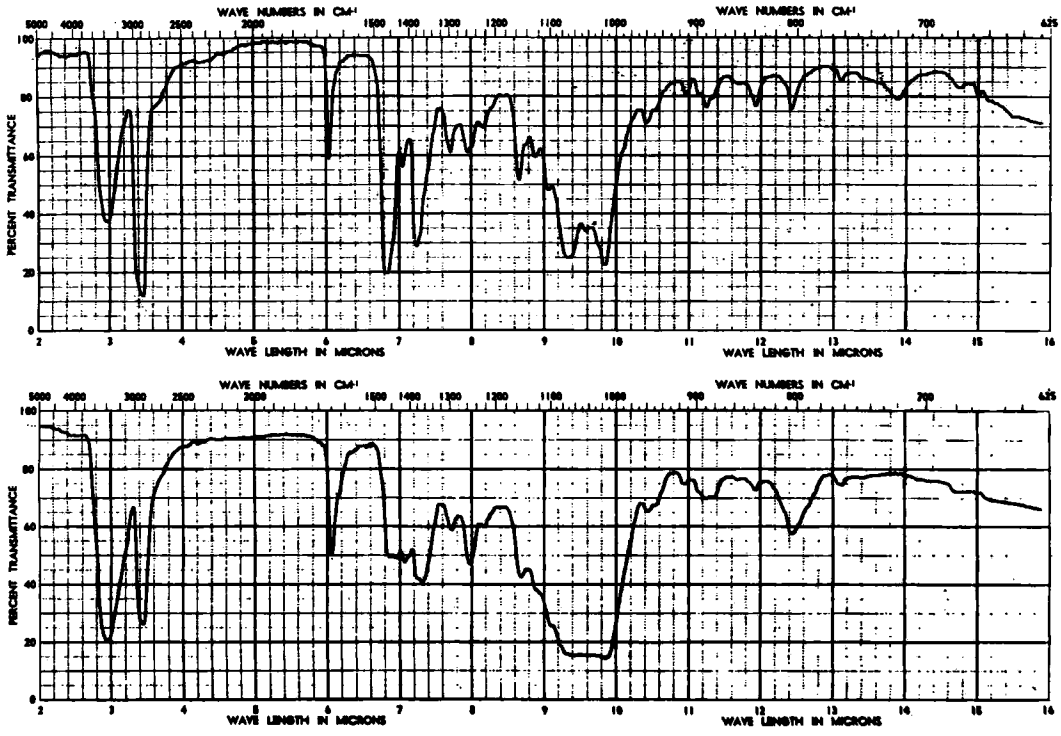

Fig. 5.—(*a*) Infrared spectrum of sabadene in Nujol. (*b*) Infrared spectrum of sabadene in a potassium bromide pellet.

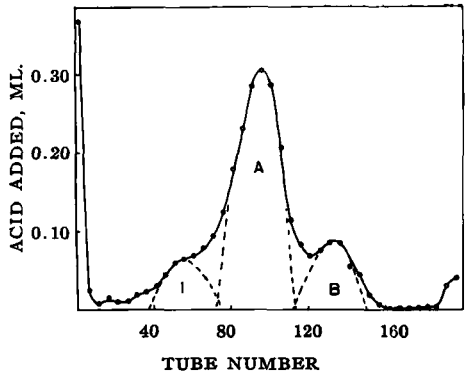

Fig. 6.—Countercurrent distribution of a 1-Gm. sample of Fraction II at pH 6.70 for 297 transfers *vs.* chloroform.

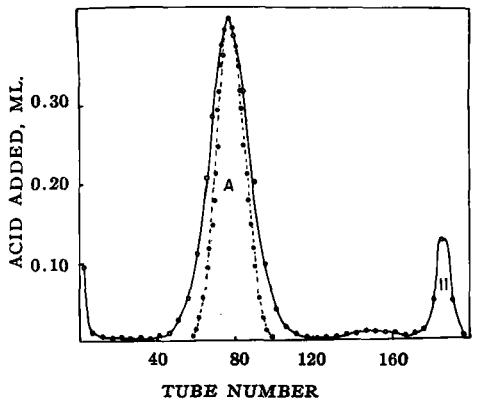

Fig. 7.—Countercurrent distribution of Peak A, Fraction II at pH 6.70 for 204 transfers *vs.* chloroform. Key: O—experimental, ⊖—theoretical.

sabadene from acetone-water. Crystalline sabadene melted from 218 to 219.5° after drying at 0.01 mm. for 24 hours. The silky mass of white needles lost their shiny appearance upon drying.

Elemental analyses best agreed with the formula $C_{28}H_{41-45}O_7N$. Insolubility of the material in chloroform required that the infrared spectrum be determined in Nujol (Fig. 5a) or as a potassium bromide pellet (Fig. 5b). Sabadene has a calculated A.S. value of 6.22.

Constituents of Fraction II.—Distribution of Fraction II (1-Gm. samples) at pH 6.70 for 200–300 transfers *versus* chloroform resulted in the resolution of three peaks (Fig. 6). Peak I, Fraction II, had a calculated A.S. value of 6.16 which suggested that it consisted of sabadene (6.22). Peak B, Fraction II, had a calculated A.S. value of 6.76 which suggested its identity as sabadilline II (6.72). Peak A, Fraction II (calculated A.S. value = 6.50), appeared to be another new alkaloidal constituent. The tube contents which constituted Peak A, Fraction II, were collected and distributed at a pH of 6.70 for 204 transfers *versus* chloroform (Fig. 7). Peak A, Fraction II, had a calculated A.S. value of 6.42 and Peak II, 7.64. The infrared spectrum of Peak II was identical to that of authentic veracevine although the A.S. values did not agree (7.64 *versus* 8.15). However, Peak II could only have resulted from the distribution conditions. This suggests that the alkamine of Peak A, Fraction II, is a veracevine type. The melting characteristics of amorphous Peak A, Fraction II, were as follows: swelled from 128–134°, melted 152–157°, remelted 152–157°, and decomposed slowly from 230–290°.

Constituents of Fraction III.—The distributions of Fraction III (1.0-Gm. samples) at pH 6.65 *versus* chloroform resulted in the isolation of sabadilline II from Peak A, Fraction III, and what appeared to be its alkaline isomerization product (Peak B, Fraction III) which has been discussed previously (2). In addition, three very minor distribution peaks were observed. Peak I, Fraction III (A.S. value 6.52), was probably identical to Peak A, Fraction II (A.S. value 6.50). Peak II, Fraction III (A.S. value 7.23), was probably identical to sabatine (A.S. value 7.33). Peak III, Fraction III (A.S. value 7.56), was probably identical to Peak A, Fraction V (A.S. value 7.51).

Constituents of Fraction IV.—The material which comprised Fraction IV had the same calculated A.S. value (7.33) as did sabatine. Crystalline sabatine

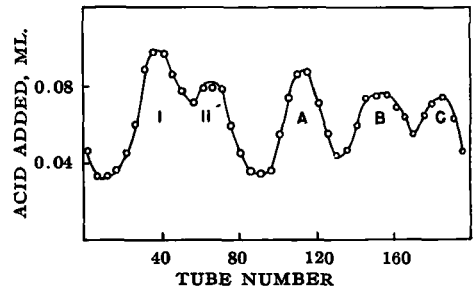

Fig. 8.—Countercurrent distribution of a 500-mg. sample of Fraction V at pH 7.80 for 210 transfers *vs.* chloroform.

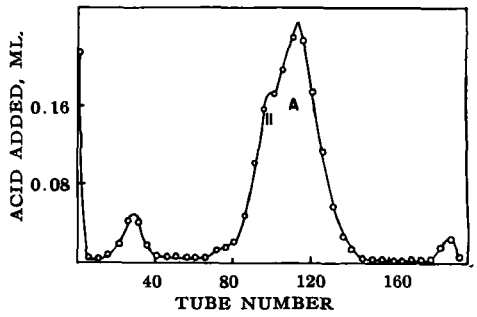

Fig. 9.—Countercurrent distribution of Peak A, Fraction V at pH 7.50 for 215 transfers *vs.* chloroform.

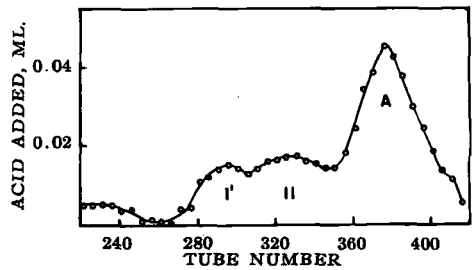

Fig. 10.—Countercurrent distribution of Peak A and Peak II, Fraction V at pH 7.50 for 697 transfers *vs.* chloroform.

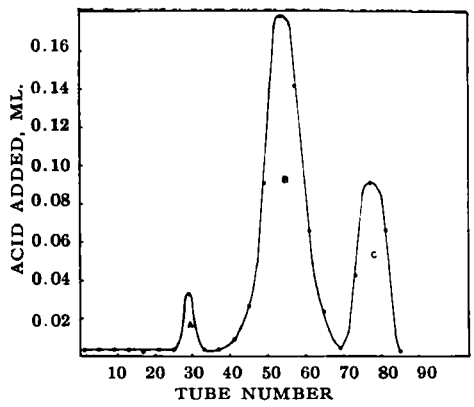

Fig. 11.—Countercurrent distribution of Fraction VI at pH 8.00 for 90 transfers *vs.* chloroform.

was isolated from this peak material. In addition, Fraction IV exhibited infrared absorption characteristics similar to sabatine.

Constituents of Fraction V.—The conditions which resulted in the best resolution of 0.5–1.0-Gm. samples of Fraction V were a pH of 7.80 and about 210 transfers *versus* chloroform. A typical distribution pattern obtained is shown in Fig. 8. Peak I, Fraction V, had an average calculated A.S. value of 6.77 which corresponded to the A.S. value of sabadilline II (6.72). Peak II, Fraction V, had an average A.S. value of 7.52. Peak A, Fraction V, appeared to be composed of at least two constituents in one distribution (not shown), but was generally obtained as a single symmetrical peak. The material which comprised Peak A and Peak II was collected and distributed at pH 7.50 for 215 transfers *versus* chloroform. As can be noted from Fig. 9, a poorly resolved peak of A.S. value 7.43 was obtained. This was believed to be similar to Peak II, Fraction V. The material which constituted Peak A was again collected and distributed at pH 7.50 for 697 transfers *versus* chloroform (Fig. 10). All of the alkaloidal material was contained in tubes 220 to 400. Again, material other than Peak A was observed. These minor peaks, I and II, were not further investigated. Amorphous Peak A (A.S. value 7.51) was collected and the following melting characteristics were obtained: swelled 105–115°, melted 140–150°, reswelled and became opaque from 222–229°, decomposed slowly above 310.

As noted previously (2), Peak B, Fraction V, was found to consist of sabadilline III.

Peak C, Fraction V, had an average calculated A.S. value of 8.12. This corresponded quite closely to the A.S. value of veracevine (8.15). All attempts to crystallize this material were unsuccessful. The infrared spectrum of Peak C was identical to that of authentic veracevine.

Constituents of Fraction VI.—A single distribution of Fraction VI (100-mg. sample) was made at pH 8.00 for 90 transfers *versus* chloroform. Three peaks were obtained (Fig. 11). Peak A had a calculated A.S. value of 7.68, but the small amount of material obtained precluded further study. Peak B had a calculated A.S. value of 8.15 and was undoubtedly veracevine (A.S. value 8.15). Peak C had a calculated A.S. value of 8.79, which is intermediate between the values for cevagenine (8.6) and sabine (8.9). Peak C was not further investigated.

TABLE I.—TOTAL KNOWN AND UNKNOWN ALKALOIDAL COMPOSITION OF SABADILLA

Compound	Source (1, 2)	A.S. Value
Veratridine	$P_{6,7,8}$	3.65
Cevadine	$P_{6,7,8}$ $P_{3,4,5}$	4.27
Vanilloyl veracevine	$P_{3,4,5}$ $P_{6,7,8}$	4.87
Unknown	Peak I, Fraction I	5.00
Unknown (mixture of three constituents)	Peak II, Fraction I	5.47
Cevacine	Peak A, Fraction I	5.88
Sabadilline I	Peak B, Fraction I	6.05
Sabadene	Peak C, Fraction I	6.22
Unknown (single constituent)	Peak A, Fraction II	6.42
Sabadilline II	Peak A, Fraction III	6.72
Alkaline isomerization product of Sabadilline II	Peak B, Fraction III	6.98
Sabatine	Fraction IV	7.33
Unknown	Peak I, Fraction V	7.43
Unknown	Peak II, Fraction V	7.52
Unknown	Peak A, Fraction V	7.51
Sabadilline III	Peak B, Fraction V	7.83
Veracevine	Peak C, Fraction V Peak B, Fraction VI	8.15
Cevagenine	.....	8.6
Unknown	Peak C, Fraction VI	8.79
Sabine	.....	8.9

Pharmacological Evaluation of Sabadene.—In the dog under phenobarbital anesthesia doses up to 1 mg./Kg., intravenously, this had no significant effect on blood pressure, respiration, gut motility, or EKG.

SUMMARY AND CONCLUSIONS

In addition to the previously known sabadilla alkaloids, the three sabadillines and the aqueous isomerization product of sabadilline II, the six fractions of the hydrophilic sabadilla alkaloids have been shown to contain additional minor alkaloids. The total known and unknown alkaloidal composition of sabadilla is presented in Table I in increasing order of A.S. value.

It is possible that Peak C, Fraction VI, may consist of several materials which include cevagenine and sabine, although these materials were not directly noted in this study.

A procedure such as cited in our several papers on the countercurrent separation of the sabadilla alkaloids is to be recommended in the analysis of complex mixture of natural products which possess slightly different partition characteristics. Isolation of products in crystalline form or known chemical identity is not a prerequisite to identification of the constituents.

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Dissolution Behavior of Crystalline Solvated and Nonsolvated Forms of Some Pharmaceuticals

By ELI SHEFTER† and TAKERU HIGUCHI

Relative rates of dissolution of several crystalline steroids, xanthines, and other solid drugs have been measured to determine the effect of solvate formation on this property. Equations have been derived relating the solubility products and diffusional constants to rates of solution of organic solvates. The results suggest that the tendency of many drugs to form such adducts provide pharmaceutical investigators a powerful tool in effecting rapid dissolution of highly insoluble substances.

MANY ORGANIC and inorganic compounds are capable of existing in more than one crystalline form having different physical properties. In some cases these states are the result of solvate formation; in others the molecular arrangement within the crystal lattice is responsible for the differences in the properties of crystal modifications. The pharmaceutical importance of variations in the thermodynamic properties associated with the differences in crystal forms have been recently pointed out. It has been suggested by Higuchi (1) that the differences in the free energy of polymorphs, for example, can be an extremely important factor in determining the stability and availability of certain pharmaceuticals. The present report is concerned with the results of theoretical and experimental studies conducted to determine the magnitude of the differences in the thermodynamic and dissolution properties arising from the formation of such crystalline variants of a drug.

Organic medicinal compounds appear to be particularly prone to both formation of polymorphs and solvates and to have large differences in energy associated with such crystalline modifications. This can be ascribed to the size and general complexity of molecules having medicinal value and to their polyfunctionality. Such structures do not usually favor rapid crystalline nucleation and growth. These forms which are favored from the rate of crystal growth viewpoint often turn out to be metastable compared to more difficultly produced polymorphs. It is apparent that the appropriate selection of the most suitable crystalline modification, whether arising from polymorphic differences or as a result of solvate complex formation, can often significantly increase the medicinal value of a given drug in a particular dosage form.

Specifically, this study was concerned with a theoretical and experimental investigation of the relative dissolution rates of some crystalline solvates and polymorphs in aqueous solutions. The thermodynamic properties of these crystal systems also have been experimentally determined in several instances.

The six crystalline systems whose dissolution behaviors were studied are cholesterol, forms N_1 and H_1; caffeine, forms N_1 and H_1; theophylline, forms N_1 and H_1; glutethimide, forms N_1 and H_1; succinyl sulfathiazole, forms N_1, H_1, H_2, and P_1; and 9α fluorohydrocortisone acetate (fludrocortisone acetate), forms N_1, N_2, P_1, and E_1, where N_1 represents a nonsolvated crystalline form and H_1, P_1, and E_1, represent a specific hydrate, pentanol solvate, and ethyl acetate solvate, respectively, for each compound. Polymorphic and additional variations are noted by appropriate subscripts. The thermodynamic differences between the various solvated and nonsolvated crystal forms of theophylline, glutethimide, fludrocortisone acetate, and succinyl sulfathiazole were evaluated. In addition, studies were undertaken to determine the effect of certain protective colloids on prolongation of a metastable phase.

PAST WORK ON CRYSTALLINE HYDRATES AND SOLVATES

Past studies on the physical chemical properties of crystalline hydrates appear to have been mainly limited to inorganic compounds. The phase solubility method was utilized, for example, by many workers to obtain the solution properties of inorganic hydrate systems in aqueous solutions. The investigation of Taylor and Henderson (2) on the various hydrates of calcium nitrate is a good example of the type of results obtained by this method.

Hill (3), in a similar type study on calcium sulfate, was able to determine accurately the transition temperature between an anhydrous form and a dihydrated form. Kuznetsov and co-workers (4) determined some of the factors involved in the con-

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version of an anhydrous form of calcium sulfate to the dihydrate at 18°.

Eriksson (5) recently examined the apparent solubility of an anhydrous form and two hydrates of phenobarbital as a function of time in water. The concentration of drug in solution was measured on an hourly basis, assuming no immediate conversion of the metastable phases in the solution. In this investigation the solubility of the anhydrous form was greater than the two hydrates below 50°. No attempt was made to evaluate the thermodynamic properties or dissolution rates of these crystalline modifications. This study seems to be the only investigation reported in the literature which compares the solubilities of hydrated and anhydrous forms of organic compounds.

There are many references in the literature on the formation of crystalline solvates of organic compounds. These references are usually in connection with organic synthesis, a particular compound being reported as crystallizing from an organic solvent with molecules of the solvent attached. No previous investigations seem to have been reported on the properties of behavior of these types of solvates in aqueous media.

THEORETICAL CONSIDERATIONS

A general discussion of the theories basic to the equilibrium solubility (thermodynamic) and rates of solution for the various types of crystal systems studied is presented.

Thermodynamic Considerations

The equilibrium solubility of a nonsolvated form of a crystalline, nondissociating organic compound, A, in water may be represented by equilibrium 1 (Eq. 1) (6). This equilibrium is not only in-

$$A_{solid} \overset{Ks}{\rightleftharpoons} A_{aqueous} \qquad \text{(Eq. 1)}$$

fluenced by the parameters of temperature and pressure, but also by the crystalline state. Because organic compounds are able to exist in more than one crystalline state (polymorphism), it is necessary to identify the solid state in this equilibrium as a parameter.

The equilibrium constant, Ks, at a particular temperature and pressure may be defined as the solubility of a particular polymorph and is approximately proportional to the thermodynamic activity of the solid. An extensive treatment of the thermodynamic differences in the properties of polymorphs can be found in a recent report by Higuchi, *et al.* (7).

Equilibrium 2 (Eq. 2) represents the solid-solution

$$A:xH_2O_{solid} \overset{Ks}{\rightleftharpoons} A_{aqueous} + xH_2O \qquad \text{(Eq. 2)}$$

equilibrium for a hydrate in water. This system is analogous to the equilibrium for the nonsolvated form; consequently, the equilibrium constant, Ks, is essentially the solubility of the hydrate.

The hydration of an anhydrous crystal modification in water is represented by Eq. 3. The free

$$A_{solid} + xH_2O_{liquid} \rightleftharpoons A:xH_2O_{solid} \qquad \text{(Eq. 3)}$$

energy change for this process may be computed from Eq. 4

$$F_T = RT \ln \frac{(Ks) \text{ Hydrated form}}{(Ks) \text{ Anhydrous form}} \qquad \text{(Eq. 4)}$$

This energy difference can easily be obtained from solubility data for the two crystal forms at a particular temperature. Since the solubilities can be readily measured at several temperatures, the enthalpy and entropy changes corresponding to this process can also be determined. These thermodynamic data are of some theoretical interest relative to the information they provide concerning the molecular bonding of water in these solids.

An equilibrium model for the crystalline solvates of the type studied is shown in Eq. 5. This model assumes that in solution A and B exist as separate

$$A:nB_{solid} \overset{Ksp}{\rightleftharpoons} A_{aqueous} + nB_{aqueous} \qquad \text{(Eq. 5)}$$

species whose concentrations are determined by the solubility product, Ksp.

The free energy change corresponding to the process shown by Eq. 5 could be evaluated from the solubility of the solvated A in pure B, the solubility of unsolvated A in B, the solubility of the same unsolvated A in water, and the activity coefficient of B in water. It suffices to say at this point that for the solvates with which this study is concerned, Eq. 5 usually represents a far greater free energy difference than Eqs. 1 and 2.

Dissolution Rate (General Discussion)

Noyes and Whitney (8) have shown that the rate of dissolution of solids is directly proportional to the concentration gradient when the surface area of the dissolving material changes negligibly for systems yielding only a single species in solution.

In their equation, Eq. 6, Cs is the concentration of the saturated solution and Ct is the amount dis-

$$\frac{dC}{dt} = k(Cs - Ct) \qquad \text{(Eq. 6)}$$

solved at time t. The k in this equation has been shown to be dependent on many factors—on the surface area of exposed solid (9), the intensity of agitation (10), the temperature (11, 12), the size and shape of the particles (9), the apparatus, and the diffusion constant of the dissolved material. It is evident from the equation that for dissolution processes occurring in media where $Ct/Cs \ll 1$, such as may be found during the absorption of uncharged, nearly insoluble drugs *via* the oral route, the solubility term Cs is the major determining factor. This same relationship holds whether anhydrous forms or hydrates of drugs are being dissolved in water.

For systems which yield more than a single species in solution, such as the case with solvates of the type $A:nB$, the Noyes-Whitney equation cannot be used to predict their dissolution rate. The dissolution rate of a solvated organic solid in water seems never to have been treated mathematically. Since it appears that these crystalline addition complexes may possess such significantly high rates of solution as to be of pharmaceutical importance, equations relating some of the factors involved have been derived.

For a crystalline substance which dissociates as follows in water, and which follows the solubility

$$A{:}nB_{solid} \rightleftharpoons A_{aqueous} + nB_{aqueous}$$

product principle of the rate of dissolution under

$$[C_A]\ [C_B]^n = Ksp$$

constant stirring and geometric conditions may be written as

Rate of Dissolution =

$$G = \frac{dC_{A^*}}{dt} = kD_A\,(C_A - C_{A^*})$$

$$= \frac{1}{n}\frac{dC_{B^*}}{dt} = \frac{kD_B}{n}\,(C_B - C_{B^*}) \quad \text{(Eq. 7)}$$

C_{A^*} and C_{B^*} refer to the concentration of the two species in the bulk of the solution, C_A and C_B their respective concentrations at the immediate crystal surface, D_A and D_B the respective diffusitivity, and k the combined geometric and agitation factor. For our thinking, A can be considered to be the drug component (*e.g.*, steroid or sulfa drug) and B the organic solvent adduct (*e.g.*, amyl alcohol).

For the case $n = 1$ and $D_A = D_B$ the above relationships yield a very simple solution which is

$$G = \frac{dC_{A^*}}{dt} = kD_A(\sqrt{Ksp} - C_{A^*}) \quad \text{(Eq. 8)}$$

similar in form to Eq. 6. It is evident that C_{A^*} may be able to build well above the solubility of A itself in water.

Other simple relationships can be derived from the above equations when $n = 1$ but $D_A \neq D_B$. Thus from Eq. 7 and the solubility product, we have

$$G = kD_A\left(\frac{Ksp}{C_B} - C_{A^*}\right) \quad \text{(Eq. 9)}$$

And since $G = kD_B\,(C_B - C_{B^*})$

$$C_B = \frac{G}{kD_B} + C_{B_*} \quad \text{(Eq. 10)}$$

By substituting Eq. 10 into Eq. 9, the quadratic in Eq. 11 is obtained.

$$G^2 + G(kD_BC_{B^*} + kD_AC_{A^*}) - k^2D_BD_AKsp + k^2D_BD_AC_{B^*}C_{A^*} = 0 \quad \text{(Eq. 11)}$$

When $C_{A^*} = C_{B^*}$, the quadratic Eq. 11 is easily solved to yield Eq. 12

$$G = \frac{dC_{A^*}}{dt} = \frac{kC_{A^*}}{2}\times \left[\sqrt{(D_B - D_A)^2 + \frac{4KspD_AD_B}{(C_{A^*})^2}} - (D_A + D_B)\right] \quad \text{(Eq. 12)}$$

This equation leads to a zero rate only when $(C_{A^*})^2 = Ksp$ as expected and does not represent a first-order approach to saturation as is the case in both Eqs. 6 and 7.

If the concentration of C_{B^*} is increased much more than C_{A^*} by addition of the solvating solvent to the dissolution media, a binomial expansion can be used to simplify Eq. 11.

$$G = \frac{KD_AKsp - kD_AC_{A^*}}{C_{B^*}} \quad \text{(Eq. 13)}$$

which indicates that the dissolution rate for the solvate will decrease with an increase of B in solution.

Equations for solvates of higher order than one-to-one become more difficult to solve. In general the dissolution rates for these solvates are also dependent on their Ksp values.

EXPERIMENTAL

Apparatus

A glass-jacketed, 250-ml. Erlenmyer flask, constant temperature bath equipped with circulating pump,[1] magnetic stirrer, 1 1/2 in. flurocarbon-covered stirring bar, timer,[1] latex rubber tubes plugged with glass wool; Swinney hypodermic adaptor,[2] Millipore filters[2] (pore size 0.45 μ), Cary recording spectrophotometer (model 11 ms.), and Kofler melting-point apparatus[3] comprised the apparatus utilized.

Compounds

Cholesterol.—The hydrate (H_1) was obtained by crystallization of cholesterol (Eastman Kodak White Label) from an ethyl alcohol–water solution. Titration of these crystals with Karl Fisher reagent showed that 1.0 ± 0.2 molecule of water was bound to each cholesterol molecule.

Determination of the true melting point of this hydrate was not possible because of the rapid transformation of it to a nonsolvated form at elevated temperatures. This type of transformation also hindered the determination of the true melting behavior than the other crystalline forms of a compound and for the relatively stable nonsolvated forms.

The anhydrous form (N_1) (m.p. 149°) was formed by heating the hydrate above 80° for 48 hours.

Glutethimide.—The method of Neveu and Legagneur (13) was used to prepare the anhydrous and hydated forms of this compound. The hydrate (H_1) (m.p. 68°) was obtained by recrystallization of glutethimide (supplied by Ciba Pharmaceutical Co.) from water. The anhydrous crystals (N_1) (m.p. 83°) were prepared by recrystallization of the hydrate from anhydrous ethyl ether.

Theophylline.—The monohydrate (H_1) was obtained by using water to recrystallize theophylline (U.S.P. grade). The anhydrous form (N_1) (m.p. 272°) was prepared from the hydrate by heating it at 100° for 24 hours (14). Characteristic X-ray powder diffraction patterns (Debeye-Scherrer) for these two forms have been obtained.

Caffeine.—The monohydrate (H_1) and an anhydrous form (N_1) (m.p. 238°) of this compound were prepared in the same manner as that used for preparing the two forms of theophylline. Characteristic X-ray powder diffraction patterns (Debeye-Scherrer) of these two crystal forms have been obtained.

Fludrocortisone Acetate.—The crystalline pentanol solvate (P_1) and ethyl acetate solvate (E_1) of this compound were obtained by recrystallization of this steroid from the respective solvent. A nonsolvated crystal form (N_1, form 1) (m.p. 218–222°) was prepared by crystallization from a dilute ethyl alcohol solution.

The amount of solvent bound in each crystal form was determined by comparing the molar absorptivities of the different forms. The pentanol solvate

[1] Obtained from Precision Scientific Co., Chicago, Ill.
[2] Obtained from Millipore Corp., Bedford, Mass.
[3] Obtained from Arthur H. Thomas Co., Philadelphia, Pa.

contained 1.1 molecules of pentanol for each molecule of steroid. The ethyl acetate solvate was found to have 0.5 molecule of solvent bound to each molecule of fludrocortisone acetate.

A nonsolvated form (N_2, form 2) was isolated after the normal amyl alcohol solvate was agitated in an aqueous solvent for 24 hours and had the same molar absorptivity as form 1.

Characteristic X-ray powder patterns for each of these crystal forms have been obtained.

Succinyl Sulfathiazole.—A monohydrate (H_2, form 2) was obtained by recrystallization of succinyl sulfathiazole (obtained from National Biochemical Corp.) from a 25% ethyl alcohol–water solution. In the dissolution studies on this hydrate, another hydrate (H_1, form 1) was isolated. An anhydrous form (N_1) (m.p. 188°) of this compound was prepared by drying the crystals of form 2 to 100° *in vacuo* for 24 hours.

The pentanol solvate (P_1) (partial melting with subsequent resolidification between 127 and 136°, followed by complete melting at 191°) was obtained by recrystallization of the anhydrous form 1 from hot pentanol. By comparison of the molar absorptivity of this crystalline form with that of the nonsolvated form (N_1) it was found that 0.9 molecule of pentanol was bound to each molecule of the sulfa drug.

The distinctly different X-ray diffraction patterns for these four crystalline modifications have been obtained.

General Procedure in Dissolution Studies

The general procedure used to study the dissolution properties of all the crystal systems was similar. For each series of dissolution studies at a particular temperature for a drug, the same weight of each crystalline modification was used. The amount of the solid used was approximately in fourfold excess of that necessary to saturate the selected solvent with the most soluble form. The weighed sample was added rapidly to exactly 200 ml. of the solvent in a thermostated 250-ml. Erlenmyer flask subjected to agitation as described below. At measured time intervals small samples were withdrawn from the system and filtered. These samples were then analyzed for the concentration of drug present by an appropriate method.

Several of the specific procedures describing the individual steps in the above procedure are given in more detail in the following sections.

Agitation

The solution in the flask was agitated by a fluorocarbon-covered magnetic stirring bar rotating at a high speed. Although no serious attempt was made to maintain the intensity of agitation exactly constant in all the dissolution experiments, the simple procedure appeared to have given surprisingly good reproducibility of the dissolution curves for a given system.

Crystal Size

The size of the crystals in all the dissolution experiments was not controlled. Microscopic examination of many of the crystal samples showed that the initial particle size was well above 5 μ. Particles in this range should not show a solubility effect due to surface free energy (15).

Sampling

The samples from the dissolution system were withdrawn directly through a latex tube filled with glass wool. This produced a particle-free sample for analysis.

In the dissolution studies on fludrocortisone acetate and succinyl sulfathiazole, the crystal size of the various forms became smaller with prolonged agitation and were not able to be filtered by the above method. It was necessary to use a filter with a fine pore size to get good filtration. Millipore filters, pore size 0.45 μ, were used.

In the latter cases a syringe was used to withdraw the sample from the dissolution flask. A Swinney hypodermic adaptor with a Millipore filter inside was then placed on the syringe and the sample was pushed through the filter. The filtrate obtained in this manner was very clear.

Analytical Methods

The solutions of all the compounds except cholesterol were analyzed spectrophotometrically in the ultraviolet region. The concentration of cholesterol in solution was determined by a colorimetric assay (16), based on its color formation with iron in sulfuric acid.

Solvents Used

The dissolution experiments on the various crystalline modifications were generally performed in aqueous media. Since some of the compounds have a solubility in water too low to permit convenient determination of the solution concentration, ethyl alcohol–water solutions were used. In the case of cholesterol a glycerin–ethyl alcohol mixture containing approximately 5% water was used as the solvent.

In the dissolution studies on the various forms of succinyl sulfathiazole an acidic solution was used so that the ionization of this compound in water was held to a minimum.

Following is a list of the compounds studied with the solvents used, enclosed in the brackets: theophylline [water], caffeine [water], cholesterol [50% solution of glycerin in ethyl alcohol (contains approximately 5% water), fludrocortisone acetate [4.5% (v/v) ethyl alcohol in water and 15% (v/v) ethyl alcohol in water], glutethimide [13.4% (w/v) ethyl alchol in water], and succinyl sulfathiazole [~0.001 *N* sulfuric acid solution].

RESULTS AND DISCUSSION

The results of these investigations are considered in two parts. The first is concerned with the studies on the relative dissolution properties of hydrated and nonsolvated crystal systems. The second section presents the dissolution behaviors of crystalline solvates other than hydrates.

Comparison of Crystal Hydrates with Their Nonsolvated Forms

Dissolution Behavior.—The dissolution behaviors of hydrated and nonsolvated forms of cholesterol, theophylline, caffeine, glutethimide, and succinyl sulfathiazole are shown in Figs. 1–5, respectively. These figures show the concentration for each crystalline form attained in solution as a function of time

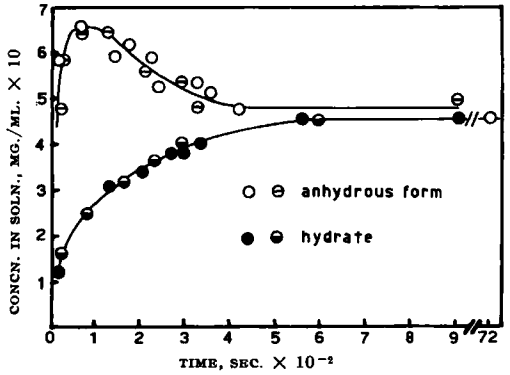

Fig. 1.—The dissolution curves for the anhydrous (N_1) and hydrated (H_1) crystalline forms of cholesterol in a 50% glycerin in ethanol solution at 25°C. The two different types of circles for each form represent successive experimental runs.

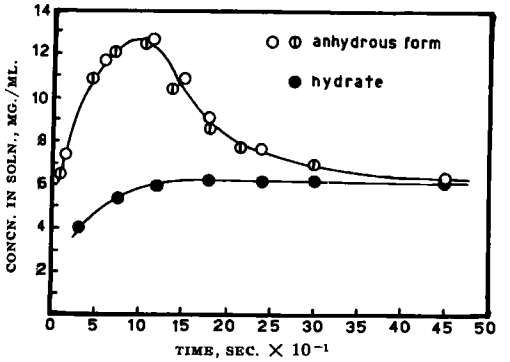

Fig. 2.—The dissolution curves for the anhydrous (N_1) and hydrated (H_1) crystalline forms of theophylline in water at 25°C. The two types of circles for the anhydrous form represent successive experimental runs.

in the presence of an excess of the solid phase and under essentially constant agitation. As indicated in some of these plots, each curve is drawn through points obtained during more than one run. Despite the relative simplicity of the dissolution technique employed, the results indicate that the procedure yielded a surprisingly reproducible rate value for each system.

It would be quite improper, of course, to attribute the apparently greater dissolution rates observed for the anhydrous forms entirely to the higher free energy contents of these species. In these as well as other dissolution experiments performed, no serious attempts were made to obtain samples possessing comparable specific surface areas, although gross microscopic examination showed no substantial differences in the size between the crystal systems of any one compound. It is evident from Eq. 6 that the ratio of the dissolution rates equals

$$\frac{\text{Dissolution Rate for Anhydrous form}}{\text{Dissolution Rate for Hydrate}} = \frac{k_A(Cs - Ct)_A}{k_H(Cs - Ct)_H}$$

where subscripts A and H represent the anhydrous and hydrated species of a particular compound. It is obvious from this ratio that some part of the observed enhancement may be due to contributions from the geometric factors k_A and k_H. It should be noted, however, that since the solubilities of the anhydrous forms in these particular instances were apparently substantially greater than those of the hydrates, the ratio of the dissolution rates of the two forms are always in the same direction as those predicted on solubility alone.

From a practical viewpoint it is evident that the rates of dissolution of a variety of crystalline drugs can be greatly influenced by the selection of the more energetic species. As seen in Figs. 1–5 the anhydrous samples dissolve much faster than the corresponding hydrates and in all cases yield concentrations substantially supersaturated with respect to the stable form. The maximal values observed in some instances may correspond to the solubility of the anhydrous crystalline phase; in others they probably represent a short-term steady-state phase situation involving equal rates of dissolution of the metastable form and crystallization of the stable hydrate.

From Fig. 1, for example, it would appear that the initial rate of dissolution of the anhydrous form, N_1, of cholesterol was very much greater than that for the hydrate, H_1, at 25°. The particular study was carried out in a 50% glycerin in ethyl alcohol solution containing approximately 5% water since cholesterol was extremely insoluble in water and because the high viscosity produced by glycerin reduced the initial dissolution rate sufficiently to permit its ready measurement. Although the maxi-

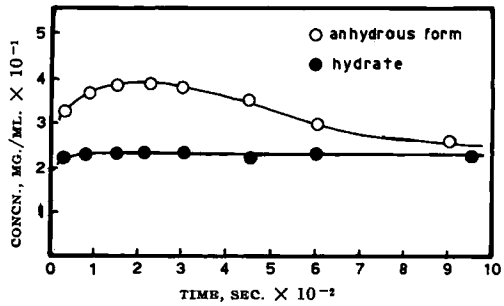

Fig. 3.—The dissolution curves for the anhydrous (N_1) and hydrated (H_1) crystalline forms of caffeine in water at 28°C.

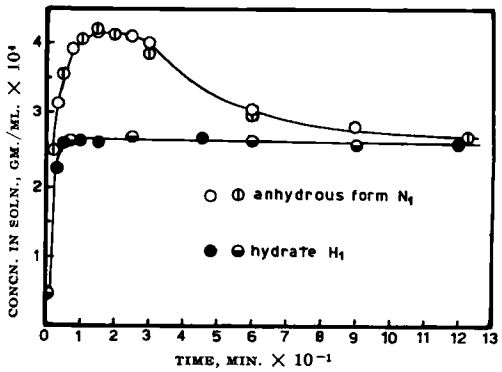

Fig. 4.—The dissolution of anhydrous and hydrated glutethimide in a 13.4% ethanol solution at 25°C. The different circles for each form represent successive experimental runs.

mum concentration reached with the nonsolvated form was only 1.4 times as great as the solubility of the hydrate, the initial rate appears to be at least three times that for the hydrate. The dissolution behavior of the anhydrous material suggests that nucleation and formation of the more stable hydrate prevented realization of the true solubility of the metastable form. It would appear that if this had not occurred, a markedly higher concentration would have been reached with the anhydrous crystals.

Qualitatively, both the dissolution and crystal growth phases appear from these diagrams to be kinetically controlled by the concentration gradient near the dissolving or growing crystalline surfaces. This is particularly evident for theophylline in Fig. 2. If the data shown are plotted as a first-order approach to equilibrium (concentration gradient controlled process) in the usual logarithmic fashion, a fairly respectable straight-line relationship is obtained. Apparently for this system the maximum exhibited by the anhydrous form, N_1, closely approaches the true solubility.

The apparent dissolution rate of anhydrous theophylline again appears to be much greater than that for the hydrate. The maximum concentration value attained with the nonsolvated crystals was twice the solubility of the hydrate at 25° in water. The higher thermodynamic activity associated with the anhydrous form apparently was the major contributing factor causing the initially greater dissolution rate observed for this modification. After the maximum concentration peak was reached with the anhydrous crystals, there is an apparent first-order decline in the amount of drug dissolved. The limiting value of this decrease was found to be the solubility of the hydrate, H_1.

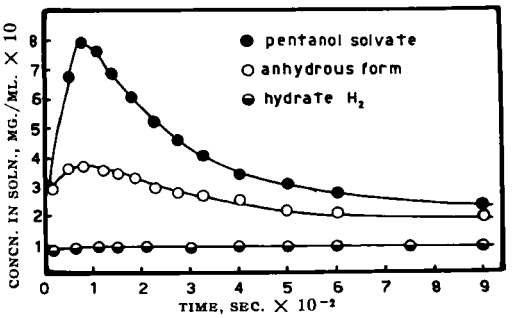

Fig. 5.—The dissolution behavior of anhydrous (N_1), hydrated (H_2), and pentanol solvated (P_1) forms of succinyl sulfathiazole in ~ 0.001 *N* sulfuric acid solution at 20°C.

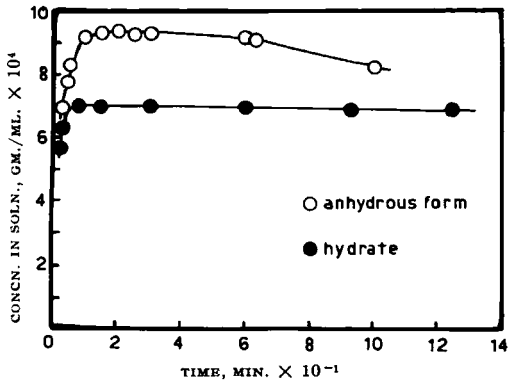

Fig. 6.—The dissolution behavior of anhydrous (N_1) and hydrated (H_1) forms of glutethimide in 13.4% ethanol solution at 40°C. Compare with Fig. 4.

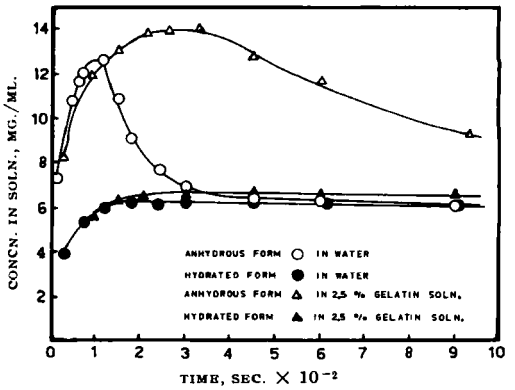

Fig. 7.—The influence of gelatin on the dissolution behaviors of the anhydrous (N_1) and hydrated (H_1) forms of theophylline at 25°C.

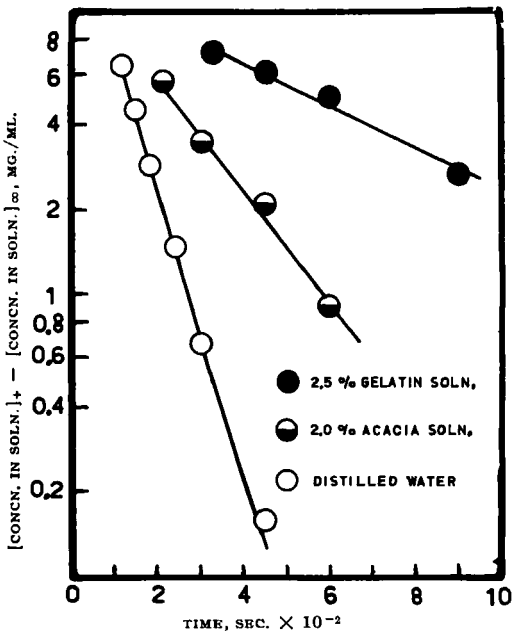

Fig. 8.—The influence of the acacia and gelatin solutions on the observed first-order transformation of anhydrous (N_1) theophylline to the hydrate (H_1) at 25°C.

Anhydrous caffeine, N_1, apparently also exhibits a greater initial dissolution rate than the hydrated form, H_1, in water. Figure 3 shows that the maximum concentration of caffeine achieved in solution with the nonsolvated form is 1.7 times the solubility

TABLE I.—INFLUENCE OF ADDITIVES ON RATE OF CRYSTALLIZATION OF THEOPHYLLINE HYDRATE

Solvent System	Transformation Rate, sec.$^{-1}$ (obtained from Fig. 9)	Water at 25°C.
Water	-5.3×10^{-3}	1.0
Acacia soln., 2.0%	-1.8×10^{-3}	1.6
Gelatin soln., 2.5%	-0.6×10^{-3}	2.5

of the hydrate at 28°. The higher concentration level reached with the anhydrous form was apparently sustained longer than that observed for many of the other metastable systems, suggesting an equilibrium saturation state over this interval.

The dissolution curves (Fig. 4) for the two crystalline forms of glutethimide also show that the initial dissolution rate of the nonsolvated form is greater than that for the hydrate. The dissolution study on this system was performed in a 13.4% (w/v) ethyl alcohol–water solution because glutethimide had a very limited solubility in pure water. The maximum concentration level attained with the anhydrous form, N_1 (1.6 times greater than the solubility of the hydrate at 25°) has a flatness similar to that observed for anhydrous caffeine. This suggests that the transformation process may occur in this system at a slower rate than, for example, cholesterol N_1. If this were the case, the plateau concentration level achieved with the anhydrous crystals might very well be its equilibrium solubility. This equilibrium effect is even more pronounced at higher temperatures as is evident in Fig. 6 for glutethimide at 40°. The observed increase in stability of the anhydrous material in solution is probably due to the reduction in the free energy difference between the two solid phases, as will be discussed later.

Succinyl sulfathiazole appears to form several hydrates and polymorphs (17). Results of the dissolution studies carried out on a few species of the sulfonamide are shown in Fig. 5. These measurements were performed in a ~0.001 *N* sulfuric acid solution to limit the ionization of the drug. The behavior of the drug in suspension strongly suggests formation of at least three hydrates. The lowest plot shows the dissolution behavior of hydrate H_2, (form 2) over a period of 15 minutes. Although an apparent equilibrium concentration is reached well within this time, storage of the suspension over several days, under the same conditions, results in a drop in the dissolved drug concentration of approximately 30% to another stable level corresponding to formation of a structurally different hydrate (H_1, form 1). The dissolution curve for the anhydrous form, N_1, shows that after the initial peak concentration value is reached, there is an apparent first-order decline in concentration to a lower constant value, but which is substantially higher than the solubility of the other two hydrates. After a few days, the

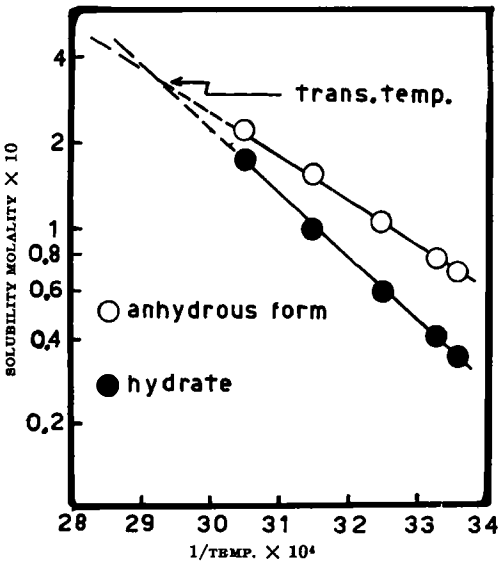

Fig. 9.—The van't Hoff-type plot for the anhydrous (N_1) and hydrated (H_1) forms of theophylline in water.

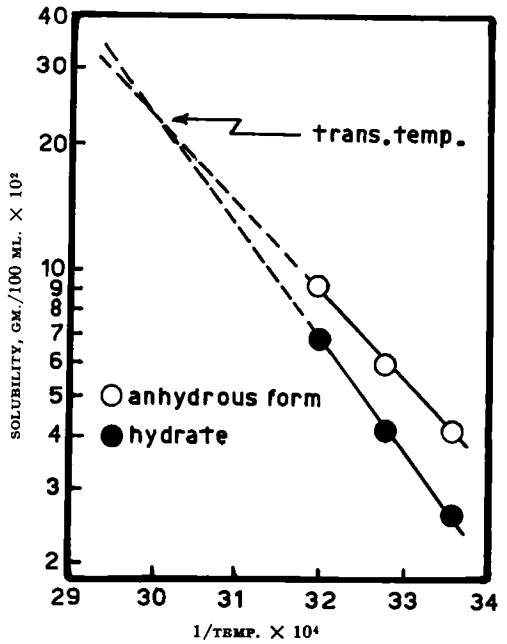

Fig. 10.—The van't Hoff-type plot for the anhydrous (N_1) and hydrated (H_1) forms of glutethimide in a 13.4% (w/v) aqueous ethanol solution.

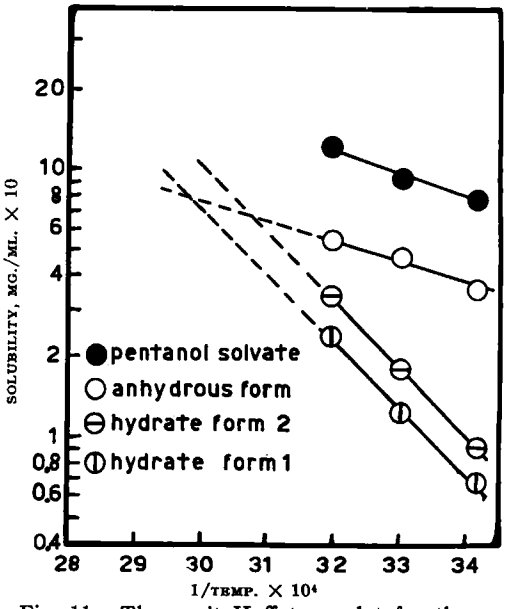

Fig. 11.—The van't Hoff-type plot for the anhydrous (N_1), hydrated (H_1), and hydrated (H_2) forms of succinyl sulfathiazole, in a ~ 0.001 *N* sulfuric acid solution. The maximal concentration of the sulfa drug attained in solution with the pentanol solvate (P_1) is included.

TABLE II.—THERMODYNAMIC VALUES CALCULATED FOR ANHYDROUS-HYDRATED SYSTEMS OF GLUTETHIMIDE AND THEOPHYLLINE

Compound	Transition Temp., °C.	ΔH, cal./mole		ΔF_{298}, cal./mole	ΔS_{298}, e.u.	$\Delta S_{Tran.\ T}$, e.u.
		Hydrate	Anhydrous			
Glutethimide	52	11,700	9700	−280	−5.8	−6.1
Theophylline	73	10,700	7400	−410	−10	−9.5

TABLE III.—THERMODYNAMIC VALUES CALCULATED FOR ANHYDROUS-HYDRATED SYSTEM OF SUCCINYL SULFATHIAZOLE

Crystal Form	ΔH, cal./mole	ΔF_{298}, cal./mole[a]	ΔS_{298}, e.u.[a]	Transition Temp. with anhydrous form, °C.
Form 1 hydrate	12,000	...	...	60
Form 2 hydrate	11,800	−210	Small + value	51
Anhydrous form (N_1)	3,400	−880	−26	...

[a] Calculated for the conversion to the most stable hydrate, form 1.

concentration level of this system decreased until it reached the solubility value of form 1 hydrate.

The results obtained for the crystalline forms of succinyl sulfathiazole are interesting from a practical viewpoint, since the compound is particularly useful in medicine because its low solubility limits its absorption in the intestinal tract. In Fig. 5 it is evident that not only is the rate of dissolution of the anhydrous form much greater than that of the hydrate form 2, but also the maximum concentration value attained is 3.9 times higher than that of the solubility of the hydrate. This may be of some practical pharmaceutical importance if the least soluble form is desired.

Effect of Protective Colloids on the Nucleation Process.—Since protective colloids are known to retard various nucleation phenomena, their effect on the transformation occurring in the present systems was investigated. Studies were carried out with gelatin and acacia to determine their influence on the rate of transformation of anhydrous theophylline to the hydrate. The study was performed at 25° in the previously described manner using water, a 2.5% gelation solution, and a 2.0% acacia solution as solvents.

In Fig. 7 the overall dissolution process observed for the two forms of theophylline in water and in 2.5% gelatin solution are compared. It is apparent that the dissolution rates of both forms were slower in the gelatin solution than in water. The slightly higher solubility of theophylline observed in the presence of gelatin may be due to an interaction with the protein. The main point of interest in Fig. 7 is that the area under the curve for the anhydrous form dissolving in the gelatin solution was much greater than observed in water.

The relative rates of formation of the hydrate species in the several solution vehicles can be best compared by plotting the approach to equilibrium as a first-order process. In Fig. 8, the solution concentrations experimentally observed minus the concentration at infinite time have been plotted as logarithmic functions against time for the precipitation phases of these measurements in water, 2.0% acacia in water, and 2.5% aqueous gelatin solution. The slopes of these lines serve as convenient measures of the relative rates of nucleation and growth of the stable phase in the several vehicles. Although the reduction in the rate of solution depletion agrees qualitatively with the increase in viscosity as measured in a capillary viscometer, quantitative fit is rather poor as is evident from Table I. It is quite likely that the effect of the protective colloid arises as the result of nucleation inhibition of both the two and three dimensional variety.

Thermodynamic Analysis for the Hydrate Systems.—The dissolution behaviors of certain of the anhydrous crystalline forms in water suggested that the maximum values obtained were good approximations of the true solubility of these crystals. It is apparent that if this were the case, that measurements made at several temperatures would permit calculations of the thermodynamic quantities involved in the transitions of the anhydrous form to the hydrate.

Dissolution plots for theophylline, succinyl sulfathiazole, and, in particular, glutethimide seem to give fairly reliable approximations of the solubility of their respective anhydrous species. Measurements over the temperature range 20 to 50° when plotted in the classical van't Hoff fashion gave reasonably good linear relationships for the anhydrous species as shown in Figs. 9–11. Similar data for the stable hydrates are also given in the same figures. The values of the heat of solution for each crystalline form, calculated from the slopes of the latter plots, appear in Tables II and III.

Enthalpy of Hydration (18).—The change in heat content for the hydration of the anhydrous crystals in water (Eq. 17) was calculated from the enthalpy changes of the following reactions.

$$A_{solid} \rightleftharpoons A_{aqueous} \qquad \text{(Eq. 15)}$$

$$A:H_2O_{solid} \rightleftharpoons A_{aqueous} + H_2O_{liquid} \qquad \text{(Eq. 16)}$$

Equation 15 minus Eq. 16 gives Eq. 17

$$A_{solid} + H_2O_{liquid} \rightleftharpoons A:H_2O_{solid} \qquad \text{(Eq. 17)}$$

The heats of reactions of Eqs. 15 and 16 are obviously the heats of solution of the anhydrous and hydrated crystals of compound A. The enthalpy change for Eq. 17, $\Delta H_{A,H}$ is therefore the heat of solution of the anhydrous form minus the heat of solution for the hydrate. $\Delta H_{A,H}$ was utilized to calculate the entropy change involved in the hydration process of Eq. 17.

The values of $\Delta H_{A,H}$ for theophylline and glutethimide were found to be −3300 and −2000 cal./-

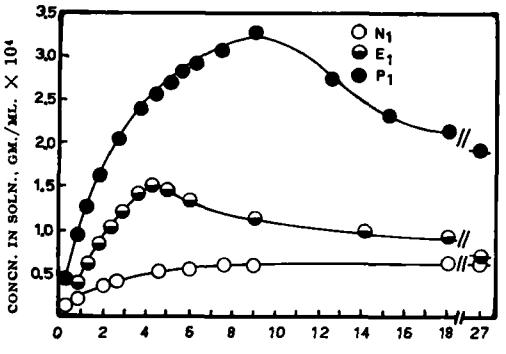

Fig. 12.—The dissolution behaviors of the nonsolvated form (N_1), pentanol solvate (P_1), and ethyl acetate solvate (E_1) of fludrocortisone acetate in an aqueous ethanol solution (15% v/v) at 20°C.

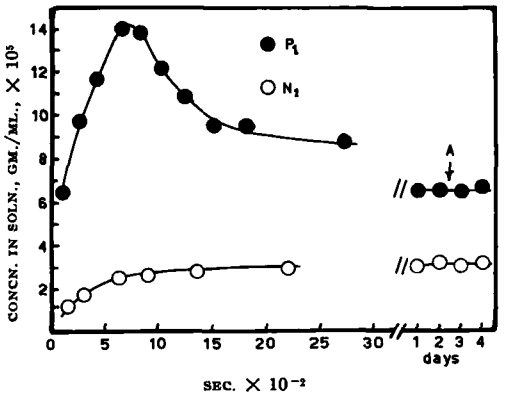

Fig. 13.—The dissolution behaviors of the pentanol solvate (P_1) and nonsolvated form (N_1) of fludrocortisone acetate in a 4.5% aqueous ethanol solution at 20°C. At the *A* concentration level, P_1 was found to be converted to a nonsolvated form (N_2).

mole, respectively. The $\Delta H_{A,H}$ value for the conversion of the anhydrous form of succinyl sulfathiazole to form 1 hydrate was −8600 cal./mole.

Free Energy of Hydration.—At constant temperature and pressure the free energy difference between the anhydrous and hydrated forms is determined by Eq. 18

$$\Delta F_T = RT \ln \frac{(Cs)\ \text{anhydrous}}{(Cs)\ \text{hydrate}} \quad \text{(Eq. 18)}$$

This equation relates the solubility, *Cs*, of the two forms at a particular temperature, *T*, to the free energy difference. This ΔF_T corresponds to the free energy change involved in Eq. 17, since the activity of water is approximately unity in the present systems. These values are listed in Tables II and III for glutethimide, theophylline, and succinyl sulfathiazole systems at 25°.

Entropy Change.—The entropy change for the hydration reaction in Eq. 17 can be calculated at a particular temperature, *T*, by Eq. 19

$$\Delta S_T = \frac{\Delta H_{A,H} - \Delta F_T}{T} \quad \text{(Eq. 19)}$$

The entropy changes for the hydration of the anhydrous crystals of glutethimide, theophylline, and succinyl sulfathiazole to their respective stable hydrate were computed by Eq. 19 at 25°. These values appear in Tables II and III.

The entropy change involved in the fusion of water at 25° is approximately −6 e.u. This decrease in entropy associated with the formation of ice is approximately the same entropy change obtained for the hydration of glutethimide and theophylline. It may be likely, therefore, that the energy involved in the transformation of the dehydrated form of these compounds to the hydrate is related mainly to the decrease in the entropy of water molecules in the hydrate structure.

The structure of theophylline hydrate was elucidated by Sutor (19). This structure analysis showed that the water molecules form a chain network throughout the lattice. One water molecule is hydrogen bonded to two other waters and to one theophylline molecule. This type of structure could be responsible for the larger decrease in entropy associated with the hydration of theophylline than with the fusion of pure water.

At the transition temperature of the anhydrous and hydrated crystalline forms, $\Delta F_{\text{Tran. } T}$ is equal to zero. Equation 19 then reduces to the simple form shown in Eq. 20. The entropy change calculated at

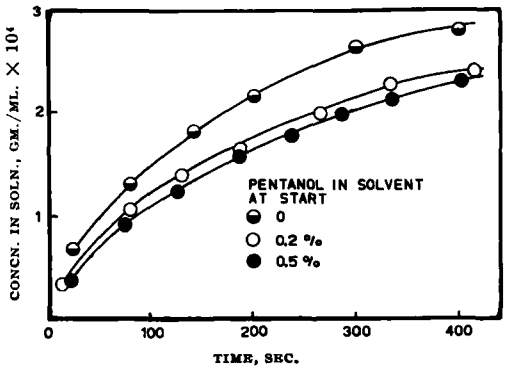

Fig. 14.—The influence of the addition of pentanol to the 15% aqueous ethanol solution on the dissolution of the pentanol solvate of fludrocortisone acetate at 20°C.

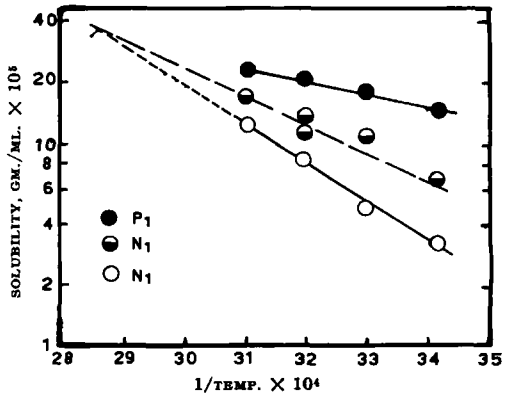

Fig. 15.—The van't Hoff-type plot for the nonsolvated forms N_1 and N_2 of fludrocortisone acetate in a 4.5% aqueous ethanol solution. The maximal concentration of steroid attained in solution with the pentanol solvate is also plotted in the same fashion.

$$\Delta S_{\text{Tran. } T} = \frac{\Delta H_{A,H}}{\text{Tran. } T} \qquad \text{(Eq. 20)}$$

the transition temperature for the theophylline and glutethimide systems are shown in Table II.

Transition Temperature.—The transition temperature for the hydrate-anhydrous crystal systems corresponds to that temperature at which the fugacity of the anhydrous form is equal to that of the hydrated form, *i.e.*, the temperature at which the solubility of the two forms are equal. The transition temperatures for the theophylline, glutethimide, and succinyl sulfathiazole systems were obtained directly from Figs. 9, 10, and 11 in that order.

Glutethimide hydrate was reported (13) by Legagneur and Neveu to decompose at 53° and melt at 68°. The transition temperature derived from the solubility data of the two crystal forms is 52°. This latter temperature could correspond to the "decomposition" temperature of the above workers.

Theophylline hydrate imbedded in paraffin and heated on a Kofler melting apparatus started to release bubbles at approximately 70°. These bubbles may indicate that water vapor is being given off by the crystals. Using the solubility of the anhydrous and hydrated forms of theophylline, a transition temperature of 73° was found.

In Table III the transition temperature for each hydrate of succinyl sulfathiazole is presented. Since form 1 hydrate seemed to be the most stable hydrate, it correspondingly had a lower transition temperature than form 2 hydrate.

Crystalline Solvate Systems

Dissolution Behavior.—The dissolution behaviors of the mono-amyl alcohol solvates of succinyl sulfathiazole and fludrocortisone acetate are recorded along with their stable modifications in Figs. 5 and 12, respectively. In the latter figure, the dissolution of the ethyl acetate solvate of fludrocortisone acetate also appears. The dissolution studies on the steroid were carried out in ethanol-water solutions because of the compounds' limited solubility in water.

These dissolution curves seemed to confirm the theoretical prediction that the concentration of drug attainable in solution with a solvate may be many times greater than that with a stable crystalline form. The pentanol solvates of the steroid and the sulfa drug produce drug concentrations five and ten times greater than the solubility of their respective stable crystalline forms. A similar measurement of the ethyl acetate adduct of fludrocortisone acetate showed that it was able to achieve more than twice the concentration in solution as did the stable form (form 1). It is apparent from the initial slopes of these dissolution curves that the solvates have, as expected, a much greater rate of solution than the stable forms.

The overall solution behavior of the pentanol solvate of the steroid is plotted in Fig. 13 at 20° in a 4.5% (v/v) ethyl alcohol–water solution. A decrease in the solubility was observed after the initially high concentration peak is reached with the solvate. This decrease corresponded to the conversion of the solvated crystals to a nonsolvated species, form 2. This was attested by X-ray analysis of the crystalline material within the dissolution flask. It appeared to have a solubility of more than twice that of form 1 at 20°. It also appears to be stable in solution at 20° for at least 5 days without subsequent transformation.

In Fig. 14, the effect on the dissolution rate of the pentanol solvate of fludrocortisone acetate by varying concentrations of pentanol is shown. The limited solubility of pentanol in the aqueous solvent allowed a maximum concentration of only 0.5% (v/v) to be used in this study. It is evident from Fig. 14 that the dissolution rate is retarded by the addition of pentanol, in qualitative agreement with Eq. 12. This equation for the generalized monosolvate indicates that the rate of solution decreases with the concentration of the solvating solvent in solution when it is present in greater concentration than the drug. An interesting result from this experiment that does not appear in Fig. 14 is that the maximum equilibrium concentration reached was the same for the three solvent systems, probably corresponding to the solubility of a nonsolvate, but was attained at different rates in the three systems. The maximum concentration peaks were attained in the following sequence: first the solvent without *n*-amyl alcohol present at 900 seconds, then the system with 0.2% (v/v) *n*-amyl alcohol at approximately 1250 seconds, and lastly the 0.5% (v/v) solution at 1800 seconds.

Temperature Dependence.—Although it was not possible to calculate the thermodynamic quantities of the solvate systems from the data obtained directly from the dissolution experiments, it is interesting to note the effect of temperature on the dissolution behaviors of the pentanol solvates. In Fig. 15, the solubility obtained for the nonsolvated polymorphs (forms 1 and 2) of fludrocortisone acetate along with the peak drug concentration attained in solution with the pentanol solvate (P_1) are shown as a function of temperature. A van't Hoff–type plot was used because it gave a relatively linear relationship for the different crystalline forms. The solubility of nonsolvate, form 2, was taken as the plateau concentration level reached after 24 hours of agitation of the pentanol solvate in solution. In Fig. 11, the maximum concentration attained with the pentanol solvate of succinyl sulfathiazole in solution is shown as a function of temperature together with the van't Hoff–type plots for the other forms of this compound that were studied.

GENERAL DISCUSSION

Experimental observations and the theoretical analysis presented above again strongly emphasize the importance of the role of the crystalline state of solid drugs. It is apparent that since the thermodynamic activity of these systems is often directly related to the physiological activity and availability, the choice of the proper crystalline form is of vital pharmaceutical concern.

We would like, in particular, to call attention to the possible broader utilization of organic solvates and complexes in dosage form development. Since the apparent free energy change associated with their dissolution in aqueous media can be vastly greater than that exhibited by an unstable polymorph relative to the stable form, much higher temporary solution concentrations and rates of solution

can be obtained by their use than from purely crystalline modification. This is because the system utilizes, in effect, the free energy of dilution of the complexing agent to raise the solubility of the drug. Since molecular complexes of this type are readily produced, particularly by relatively insoluble drugs, this approach may often provide the answer for those products which are poorly available because of slow rates of dissolution.

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Drug Standards

Determination of Amphetamine in Dosage Forms by Partition Chromatography

By JOSEPH E. MOODY, JR.

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MANY METHODS of analysis of sympathomimetic amines have been reported (1). They include direct titration of free bases, residual titration of free bases, extraction of bases combined in salts, distillation and titration of volatile bases, nonaqueous titration, gravimetric methods, ultraviolet absorption, and various chromatographic techniques. The widespread use of these preparations in medical practice and their extensive distribution in illegal channels creates a necessity for a simple and rapid method of assay.

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The proposed procedure is designed to permit the assay of small samples, thus making it applicable to the analysis of a single dosage unit. In this procedure, the extraction of the amphetamine from the dosage form is achieved by elution from an immobile aqueous phase of a column with a solution of triethylamine in chloroform. The eluate is collected in a separator containing an accurately measured quantity of sulfuric acid solution (2 in 100) into which the amphetamine is then extracted. It is quantitatively determined by ultraviolet absorption of the acid solution.

In the development of the method, several other elution procedures were investigated. In a published report of an extraction procedure for alkaloids (2), *p*-toluenesulfonic acid and the alkaloid are incorporated in an aqueous solution as the stationary phase on a partition column. Ether is used to elute the acidic and neutral

can be obtained by their use than from purely crystalline modification. This is because the system utilizes, in effect, the free energy of dilution of the complexing agent to raise the solubility of the drug. Since molecular complexes of this type are readily produced, particularly by relatively insoluble drugs, this approach may often provide the answer for those products which are poorly available because of slow rates of dissolution.

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substances without removal of the alkaloid. The *p*-toluenesulfonic acid salt of the alkaloid is then eluted with chloroform, and the eluate is passed over an alkaline column which removes the *p*-toluenesulfonic acid moiety. Several variations of this procedure were tried for dextro-amphetamine sulfate, but quantitative recoveries were not achieved.

Another of the procedures investigated employed the addition of a layer of infusorial earth with concentrated ammonium hydroxide solution to an acidic column containing the amphetamine sample. When chloroform was passed through the column, it carried through a sufficient amount of the ammonia to neutralize the acid and allow the amphetamine to be eluted as the free base. The excess ammonia was then removed by warming on a steam bath under a stream of air. The chloroform was transferred to a separator containing sulfuric acid (2 in 100) and extracted as in the method given. Though this procedure gave quantitative results, it required two more steps than the method employing triethylamine. These are the mixing and packing of the ammoniacal infusorial earth onto the column and the removal of the large excess of ammonia which is needed to permit the elution of the amphetamine from the column.

PROCEDURE

Reagents

Triethylamine.—Prepare a 1 in 200 solution of triethylamine in water-saturated chloroform. Extract 20 ml. of this solution with 10 ml. of sulfuric acid solution (2 in 100). Determine the absorbance of the acid solution at 257 mμ, using the sulfuric acid solution as the blank. If the absorbance is 0.010 or greater, purify the amine as follows.

Reflux 100 ml. of triethylamine with 20 ml. of water and 2 Gm. of sodium hydrosulfite for about 6 hours. Wash the amine with water and dry it by refluxing through a Dean-Stark trap. Finally, distill, collecting the first 75 ml. of distillate, and store it over anhydrous sodium carbonate. Test the purified amine occasionally to determine if the absorbance is below the specified maximum.

Chloroform.—Saturate reagent grade chloroform with water.

Hydrochloric Acid.—Use 1 in 100 solution.

Sulfuric Acid.—Use a 2 in 100 chloroform saturated solution.

Infusorial Earth.—Use acid washed Celite.[1]

Preparation of the Sample

Weigh and finely powder a sufficient number of tablets to provide a representative sample. Weigh accurately a portion of the powder, equivalent to about 5 mg. of dextro-amphetamine sulfate, in a 100-ml. beaker. Add 2 ml. of hydrochloric acid solution and swirl gently to wet the powder thoroughly. Warm on a steam bath for about 1 minute with occasional gentle swirling and cool. Add 3 Gm. of infusorial earth to the beaker, and mix until a fluffy mixture is obtained.

Chromatographic Column

Pack a pledget of fine glass wool with the aid of a tamping rod in the base of a chromatographic tube made from approximately 5 cm. of 6–8 mm. tubing fused to a 25 × 300-mm. test tube. The tamping rod consists of a disk of stainless steel, aluminum, or glass, with a diameter about 1 mm. less than that of the column, attached to a rod approximately 18 in. long. To 2 Gm. of infusorial earth in a 100-ml. beaker, add 1 ml. of hydrochloric acid solution and mix thoroughly with a spatula. Transfer the mixture to the column and tamp lightly to compress the material into a uniform mass. Transfer the sample preparation to the column, "dry rinse" the beaker with about 1 Gm. of infusorial earth, and transfer it to the column. Add a pledget of fine glass wool and tamp as above. Wash the column with 100 ml. of water-saturated chloroform and discard the washings.

Assay

Place a 125-ml. separator containing 10.0 ml. of chloroform-saturated sulfuric acid solution under the column to receive the eluate. Pass 35 ml. of a 1 in 200 solution of triethylamine in water-saturated chloroform through the column. Complete the elution with 70 ml. of water-saturated chloroform. Shake the separator vigorously for 1 minute, allow the layers to separate, and discard the chloroform. Scan the spectra of the acid solution of the sample and of a standard solution, containing about 0.5 mg. of dextro-amphetamine sulfate per ml., from 350 to 250 mμ in 1-cm. cells with a suitable recording spectrophotometer using chloroform-saturated sulfuric acid as the blank. Determine the baseline-corrected absorbance of the

TABLE I.—RESULTS OBTAINED IN THE ASSAY OF 5-MG. DEXTRO-AMPHETAMINE SULFATE TABLETS

Commercial Product	Per cent of Claim U.S.P. Assay	Distillation Assay	Proposed Assay
A	95.4	97.4	97.6
	95.0	97.4	97.0
B	91.2	97.6	97.8
	91.8	96.8	98.4
C	91.2	94.0	95.6
	90.2	95.6	95.6
D	96.0	97.4	97.6
	96.0	97.4	97.4
E	94.0	97.4	99.6
	94.4	97.8	98.8
F	92.0	98.0	98.4
	95.2	98.0	99.0
G	93.0	98.8	100.4
	91.6	99.0	100.0
H	82.6	94.2	93.8
	79.6	94.2	93.4
I	90.6	95.6	95.6
	91.2	95.2	96.8
J	88.8	94.4	95.0
	89.0	94.8	95.4
K	95.0	100.6	103.9
	95.0	101.0	102.8

[1] Marketed by Johns-Manville Corp., Manville, N. J.

TABLE II.—COLLABORATIVE RESULTS ON PRODUCT K OF TABLE I BY THE COLUMN-UV PROCEDURE

Collaborator	Per cent of Claim
A	102.0
	103.0
B	102.8
	102.0
C	102.0
	104.2
D	103.9
	102.8
E	103.2
	103.6

TABLE III.—RESULTS OF THE ASSAY OF OTHER SYMPATHOMIMETIC AMINES

Product	Dosage Form	Label Claim	Per cent of Claim Found
Mephentermine sulfate	Tablets	25 mg./tab.	99.0
			98.6
Ephedrine sulfate	Capsules	25 mg./cap.	100.4
			100.8
Phenylpropanol-amine HCl	Elixir	4 mg./ml.	97.5
			96.8
Methamphet-amine HCl	Tablets	5 mg./tab.	100.8
			101.2

sample and standard solutions at the maximum at about 257 mμ by drawing the baseline as a continuation of the curve between 350 and 300 mμ.

Calculate the milligrams of dextro-amphetamine sulfate per tablet by the formula $10\ C\ (A_u/A_s)(W_t/W_u)$, in which C is the concentration of the standard solution in milligrams per milliliter, A_u is the absorbance of the sample solution, A_s is the absorbance of the standard solution, W_t is the average tablet weight, and W_u is the weight of the sample taken.

RESULTS AND DISCUSSION

A simulated dextro-amphetamine sulfate tablet mixture was prepared for use in standard recovery tests to check the accuracy of the method. Duplicate assays of the mixture gave recovery values of 100.0 and 100.2%.

Eleven samples of commercial 5-mg. dextro-amphetamine sulfate tablets, were assayed by three different methods: (*a*) the U.S.P. XVI procedure, (*b*) a distillation-ultraviolet absorption procedure, and (*c*) the method described herein. A sufficient number of tablets for all three procedures was weighed, finely powdered, and the appropriate aliquot taken for each method. All of the analyses were run in duplicate, the data from which are shown in Table I.

The distillation procedure was run on the 11 samples as a check on the accuracy of the proposed column procedure. A weighed sample, equivalent to about 15 mg. of dextro-amphetamine sulfate, was distilled from a strongly alkaline solution and collected in a solution of mineral acid. The acid solution was made alkaline and extracted with chloroform. The chloroform solution was then extracted and the amphetamine measured as it is in the column method.

Product K, one of the eleven commercial samples of compressed tablets, was distributed to other chemists of this laboratory for collaborative study. The resulting data are shown in Table II.

To investigate further the scope of the method, a timed release preparation of dextro-amphetamine sulfate, consisting of encapsulated pellets, was analyzed by the column procedure. The per cent of label claim found was 103.1, 102.0, and 102.8%. The method can also be used to analyze other sympathomimetic amines with similar physical and chemical properties. The results of the analyses of four such compounds are shown in Table III.

Amphetamine has a relatively low absorptivity. Using the Beckman spectrophotometer, a concentration of the order of 0.5 mg. per ml. is required to give an optimum absorbance value for quantitative determination. Since for single-dose-unit analysis a small sample is specified, the column eluate is concentrated by extraction into an acid solution. The small quantity of triethylamine employed is sufficient to neutralize the acid and release the amphetamine from the column. When quantities of triethylamine approaching 1 ml. were used, the assay results were consistently about 2% low. The excess triethylamine reacted with the sulfuric acid to form the water-soluble amine sulfate which increased the acid solution volume and caused the low results. Levine (3), in the determination of codeine in mixtures with antihistamines, used more than 1 ml. of triethylamine in the elution. The eluate was collected in a volumetric flask and diluted to volume with chloroform from which the absorbance was determined; therefore, no interference with the concentration was encountered.

The official method of assay of dextro-amphetamine sulfate tablets U.S.P. XVI (4) was shown to have several undesirable aspects. The data in Table I show that the results from the official assays are both low and are the least precise for most of the 11 samples. The results of the distillation-ultraviolet absorption and the column-ultraviolet absorption methods are practically the same. The longer time required to complete the official assay procedure and the low assay results make its use as a general method unsatisfactory. For the varied purposes of a compendium assay, a method suitable to all available products is necessary. The proposed method meets this criterion and at the same time provides a simpler and more rapid method of assay than does the official method.

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Micro-Method for Intravenous Injection and Blood Sampling

By H. SALEM, M. H. GROSSMAN†, and D. L. J. BILBEY‡

The described methods have been found to be valuable in investigations into the body distribution and fate of pharmacological substances and are herein reported for the benefit of other workers in the field of pharmacology and toxicology.

IT HAS BEEN FOUND both expedient and practicable to combine two techniques in which substances are introduced directly into the bloodstream of small animals and recovered for determination in serial samples of blood.

The injection of prepared amounts of a substance in either solution or suspension (chemicals, drugs, bacteria, particulate matter ± radioactivity) are injected into the bloodstream of a mouse or other small experimental animal by the dorsal vein of the penis. For this purpose it has been found convenient to use a 1-ml. "tuberculin" syringe graduated into 100 divisions and fitted with a fine hypodermic needle (gauge 26–30). The animal is secured by an assistant who holds it by the loose skin behind the neck in the right hand and by either the tail or hind feet in the left in order to stretch the animal and hyperextend the vertebral column (Fig. 1). The operator then places gentle pressure over the lower abdomen and secures the tip of the penis between the thumb and forefinger of the left hand as shown in both Figs. 1 and 2. The dorsal vein of the penis is easily entered by a fine hypodermic needle and the contents of the syringe can be injected with ease. It should be noticed that in this maneuver the hands of the operator and his assistant are kept in close contact to prevent uncoordinated movements.

The advantage of this method is in its applicability to animals having no easily accessible site for intravenous injection. Although we have used the mouse to illustrate this technique, we have successfully employed it using guinea pigs and hamsters.

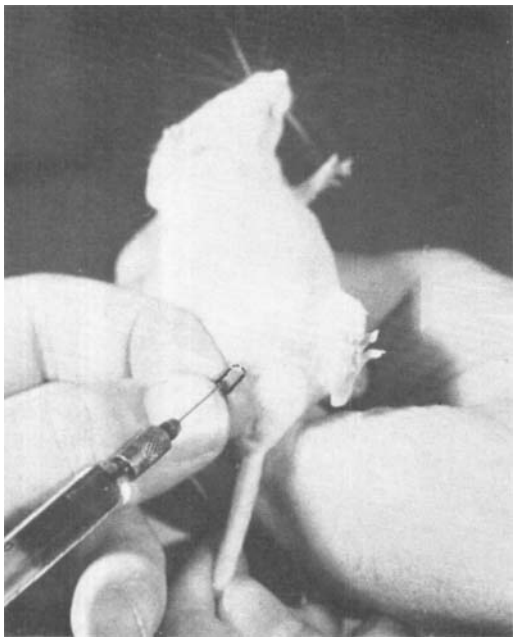

Fig. 1.—The method of holding the mouse, the retraction of the penis, and the insertion of the needle for injection into the dorsal vein.

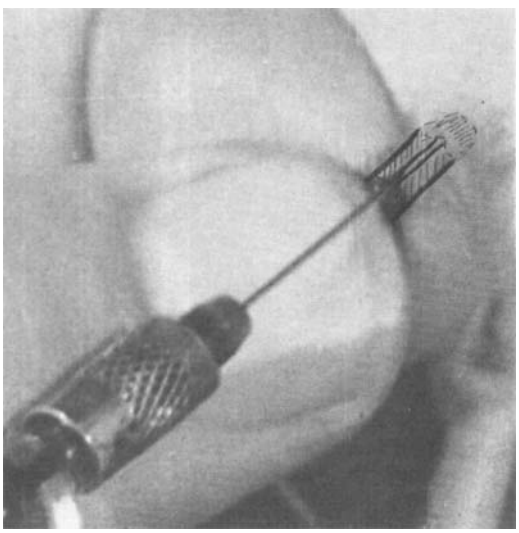

Fig. 2.—The method of insertion of the hypodermic needle into the dorsal vein of the penis. Note that the hands of the operator and assistant are in close contact to ensure stability during the injection.

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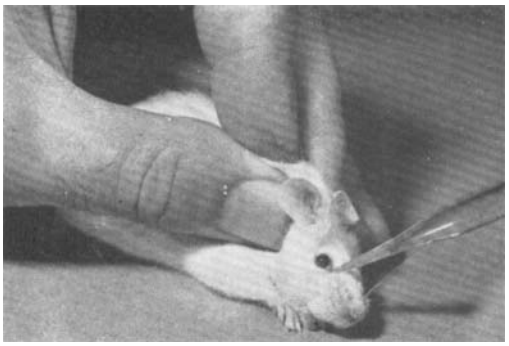

Fig. 3.—The angle at which the fine pipet should be placed at the medial canthus prior to the withdrawal of blood.

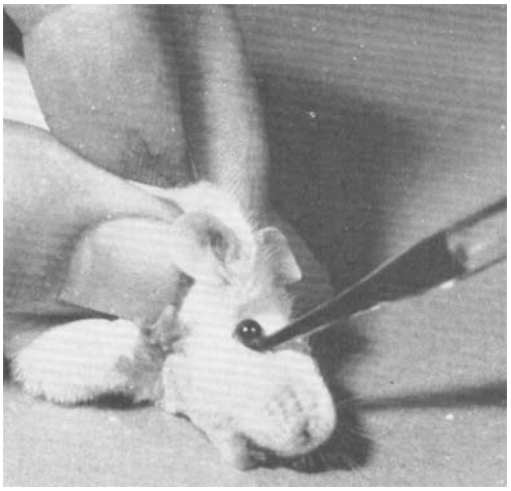

Fig. 4.—The removal of blood from the retro-orbital plexus and the positioning of the operator's thumb.

Small samples of blood are removed by the veins of the retro-orbital plexus (Fig. 5). This method has been described by Halpern and Pacaud (1) and later by Hans Nöller (2) and consists of the removal of a desired amount of the blood (usually 0.01 to 0.50 ml.) by a fine glass pipet which is inserted into the medial canthus of the orbit and which fills both by capillary action and venous pressure following the rupture of one or more of the fine branches of the orbital venous plexus. The manner of holding the animal and the angle at which the pipet is inserted is shown in Figs. 3 and 4. The middle and forefingers of

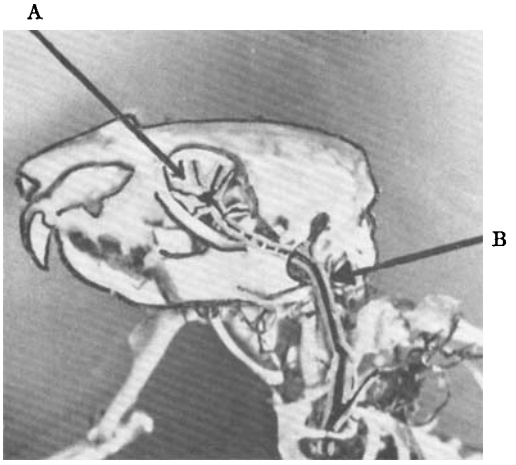

Fig. 5.—A rat skull prepared to show the veins of the head and neck in order to indicate the position of the drainage from the retro-orbital plexus of veins. *A*, indicates the orbit in which may be seen the main vessels of the plexus; *B*, shows the point at which the internal jugular vein leaves the skull and where pressure by the thumb serves to engorge the retro-orbital venous system with blood.

the left hand maintain the head of the animal in position. It is most important to press gently but firmly with the thumb just behind the angle of the jaw as shown in these illustrations. This last procedure causes a restriction of venous return by the internal jugular vein (Fig. 5) and consequent engorgement of the retro-orbital plexus. The longer the pressure is maintained, the greater volume of blood is withrawn. In order to prevent coagulation of the blood inside the pipet, the end of it is placed in a solution containing an anticoagulant before the taking of each sample. No damage to the actual eye is incurred in this procedure, and it is possible to remove up to as many as 30 serial samples from one animal. The blood that is withdrawn is available for various investigations and may be chemically, photometrically, or radioactively analyzed—cultured and plated for bacterial growth—or made into a film for observations under the microscope.

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Stability of Pancreatic Deoxyribonuclease in the Presence of Proteases

By RICHARD E. MAXWELL, VIOLET S. NICKEL, and VERA LEWANDOWSKI

Bovine pancreatic deoxyribonuclease was found to be remarkably stable in the presence of relatively high concentrations of bovine plasmin, while comparable or much lower fibrinolytic levels of chymotrypsin, trypsin, or plant proteolytic enzymes gave rapid and extensive destruction of the nuclease activity. The results are considered to illustrate a sharp divergence in substrate preference between plasmin and the other proteases.

In the evaluation of a mixture of bovine pancreatic deoxyribonuclease and bovine plasmin[1] (hereafter referred to as DNase-P) for use as a debriding preparation, the possibility that the plasmin could inactivate the deoxyribonuclease was a matter of great concern since a number of proteins may serve as substrates for plasmin (1). As no pertinent information was available in the literature, it was necessary to design a study bearing on the question; the study included comparisons with other proteolytic enzymes, particularly chymotrypsin, which was considered as an alternative proteolytic constituent of the mixture. The results are worthy of note in illustrating unexpected degrees of substrate preferences by the two proteolytic enzymes.

MATERIALS AND METHODS

Enzymes.—Vials of DNase-P were obtained from production lots. Bovine plasmin was prepared as described by Loomis, *et al.* (2), and the deoxyribonuclease was the pharmaceutical grade material supplied by Worthington Biochemical Corporation. Pharmaceutical grade α-chymotrypsin and crystalline trypsin were also obtained from the latter source.[2] Bromelin concentrate was obtained from Takamine Laboratory, ficin concentrate from Merck and Co., and papain N.F. VIII from Difco Laboratories.

Enzyme Assays.—Previously described methods were used for the determination of fibrinolytic (3), caseinolytic (4), and deoxyribonuclease (5) activities. In the case of the latter activity, results are expressed for the present purposes as the numerical decrease of relative viscosity units in the 10-minute period from the initial relative viscosity of 4.0. All solutions and dilutions of the plant enzymes contained 0.025*M* cysteine except for the final dilutions for deoxyribonuclease assay. Exposure to cysteine alone was without effect on the deoxyribonuclease.

Stability Studies.—As indicated for clinical use, vials of DNase-P were reconstituted with 10 ml. of 0.9% NaCl. Corresponding mixtures were made with the same concentration of deoxyribonuclease, and the same or different concentrations of other proteolytic enzymes as established in each case by fibrinolytic assays. Solutions were prepared at 0° and then incubated in a 37° water bath. The pH of all solutions was adjusted to 7.0, which was that of the DNase-P. At intervals during incubation identical dilutions of the various mixtures were assayed for residual deoxyribonuclease and fibrinolytic activities.

RESULTS AND DISCUSSION

As shown in Table I the level of plasmin contained in DNase-P had no effect on the deoxyribonuclease in 4 hours; the same fall in viscosity was obtained if aliquots were assayed immediately after solution of the mixture at 0°. Significant destruction of the deoxyribonuclease activity was found only when the same level of deoxyribonuclease was incubated with 8× the DNase-P level of plasmin for 4 hours. In contrast, 0.1 as much fibrinolytic activity in the form of chymotrypsin completely destroyed the deoxyribonuclease in 1 hour; even the 0.01 level of chymotrypsin gave greater loss of deoxyribonuclease in 1 hour than the 8× level of plasmin did in 4 hours.

TABLE I.—PROTEASE EFFECT ON DEOXYRIBONUCLEASE

Enzyme[a]	Initial Fibrinolytic Level	Incubation, Hr.	Deoxyribonuclease Activity Δ Vr[b]
Plasmin (DNase-P)	1.0	1	2.8
"	"	2	2.7
"	"	4	2.8
Plasmin	3.0	2	2.7
"	"	4	2.6
Plasmin	8.0	2	2.3
"	"	4	2.0
Chymotrypsin	1.0	1	0
"	0.1	1	0
"	0.01	1	1.2
Trypsin	1.0	1	0
Ficin	1.0	3	0
Bromelin	1.0	3	0
Papain	1.0	3	1.6

[a] In terms of 280 mμ absorbance, concentrations at the 1.0 level were as follows: plasmin, 6.6; chymotrypsin, 0.087; trypsin, 0.067; ficin, 4.3; bromelin, 2.1; papain, 2.7. [b] Fall in relative viscosity units in 10 minutes from the initial value of 4.0.

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[1] Marketed as Elase by Parke, Davis and Co.

[2] According to the manufacturer, pharmaceutical grade deoxyribonuclease has 70 to 90% of the activity of crystalline material. The pharmaceutical grade α-chymotrypsin has 90 to 100% of the activity of crystalline material, and trypsin contamination is "considerably" lower than one per cent.

Because plasmin is a more impure preparation than the chymotrypsin, the possibility was considered that inert proteins associated with the plasmin might protect the deoxyribonuclease from proteolytic attack. However, an equivalent amount of chymotrypsin added to the DNase-P mixture again completely destroyed the deoxyribonuclease in 1 hour. According to Feinstein and Hagen (6), ovalbumin had a remarkable effect in stabilizing deoxyribonuclease activity in crude pancreatic extracts; therefore, one per cent ovalbumin was added to a chymotrypsin-deoxyribonuclease mixture corresponding to the DNase-P formulation. The deoxyribonuclease was completely destroyed in 1 hour, indicating that the situation must be much different with the purified enzymes since this is even a higher level of inert protein than found in the DNase-P solution (about 0.6%). In this connection it might be noted that it is claimed in a recent patent (7) that solutions of crystalline deoxyribonuclease lost all activity in 3 hours at 31°. The deoxyribonuclease used in the present study showed no loss of potency in 4 hours at 37° in the presence or absence of plasmin, so that either the preparations or conditions used are not comparable in this respect. It is also now obvious that the possibility of chymotrypsin contamination must be ruled out in studies of the stability of any pancreatic deoxyribonuclease preparation.

By comparing the activities of bovine plasmin and chymotrypsin under optimum conditions of the fibrinolytic and caseinolytic assays, it was concluded that chymotrypsin "preferred" casein over fibrin as a substrate by a factor of about 3.5. In view of the above results it is apparent that the disparity may be orders of magnitude greater with respect to deoxyribonuclease (or perhaps some peptide bond related to its active center) as a substrate.

Under the conditions used for these stability studies plasmin was fairly unstable, presumably as a result of autodigestion in the absence of substrate. At the DNase-P level about 50% of the fibrinolytic activity remained after 1 hour and about 30% after 2 hours; in the case of chymotrypsin there was no significant decrease in this time. From the product processing point of view, therefore, it was possible to compensate for plasmin autodigestion by addition of excess plasmin without endangering the deoxyribonuclease activity; on the other hand, the amount of excess deoxyribonuclease required to compensate for its unexpectedly greater destruction by chymotrypsin eliminated consideration of such a mixture. The possibility of destruction of tissue deoxyribonuclease at the point of application by chymotrypsin and other proteolytic enzymes used in debriding preparations may have clinical implications, and it is hoped that information bearing on this question may be obtained in the future.

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Variation of pKa'-Values of Tetracyclines in Dimethylformamide-Water Solvents

By EDWARD R. GARRETT

The variation of pKa'-values for several tetracycline antibiotics with per cent dimethylformamide (DMF) in per cent DMF-per cent H_2O solvents (v/v) is given. Quantitative expressions for the pKa' dependence on per cent DMF for the various functional groups have been obtained. This information is utilized in the assignment of pKa'-values to functional groups.

THE DISSOCIATION CONSTANTS or pKa'-values of many of the tetracycline antibiotics have been determined in various nonaqueous-water solvents because of the difficulties of solubilization and maintenance of homogeneous solutions over the entire titratable range for one solvent alone (1–4). A frequently used solvent mixture has been dimethylformamide-water.

In addition, a traditional routine procedure has been to titrate a substance in a nonaqueous-water solvent with an aqueous titrant giving an apparent pKa' at half-neutralization difficult to compare with the results from other routine titrations, since amounts of titer may vary and the relation of pKa' with per cent nonaqueous solvent has not been established.

This communication presents information on the variation of pKa'-values for several tetracycline antibiotics, presents quantitative expressions for their dependence on per cent dimethylformamide (DMF) in %DMF–%H_2O solvents, and attempts to demonstrate their potential usefulness in deducing pKa' assignments to functional groups.

EXPERIMENTAL

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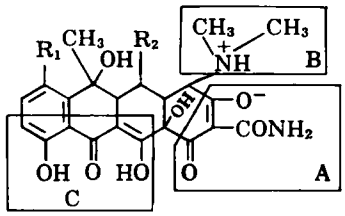
0.1000*N* HCl and 0.1*N* NaOH solutions. The titrations of desdimethylaminotetracycline and anhydrodesdimethylaminotetracycline plus added standard HCl were conducted on 10-mg. samples of the tetracyclines in 5 ml. %DMF–%H_2O solutions at 25° with 0.1000 *N* NaOH in a microburet.

The electrodes were glass-saturated calomel. The tetracycline derivatives were prepared by established methods (1–4) by Dr. Brian Bannister of the Upjohn Co.

The pKa'-values were estimated from the apparent pH values at half-neutralization of a functional group except for values *ca.* pKa' 4 and 10 where the Parke-Davis method was used (5, 6). The per cent DMF (v/v) at each pKa' was calculated from the known volume, the per cent of DMF (v/v) in the original solution and the known volume of aqueous titer added to achieve the apparent pH corresponding to that pKa'.

RESULTS AND DISCUSSION

The variation of pKa' for the various functional groups of tetracycline *Ic* as a function of the %DMF in %DMF–%H_2O (v/v) solvents is plotted in Fig. 1.



Ia, Oxytetracycline (Terramycin)
R_1 = H, R_2 = OH
Ib, Chlortetracycline (Aureomycin)
R_1 = Cl, R_2 = H
Ic, Tetracycline
R_1 = H, R_2 = H

The pKa'-assignments to the various labeled functional groups, A,B,C, of the tetracyclines *Ia*, *Ib*, *Ic* (1–4) are also designated by the lettered

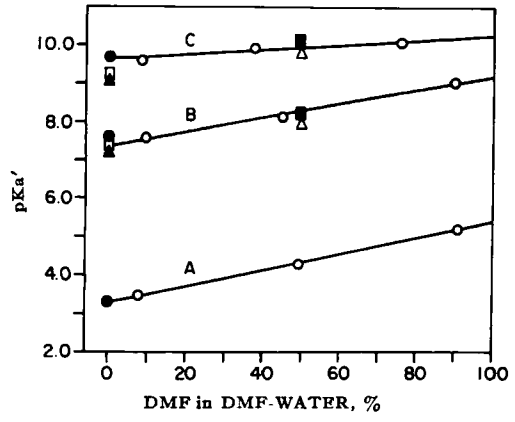


Fig. 1.—The pKa' of tetracycline *Ic* as a function of per cent dimethylformamide by volume in DMF–H_2O solvents. The open circles (○) are for the experimental data of this paper. The compounds, symbols, and references for other tetracyclines are: tetracycline, ● (4), ■ (3); oxytetracycline, △ (2), ▲ (4); and chlortetracycline, □ (4).

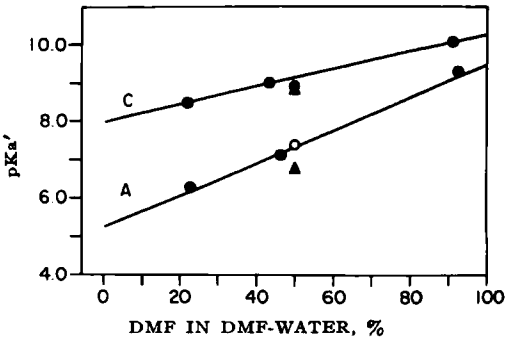


Fig. 2.—The pKa' of desdimethylaminotetracycline II as a function of per cent dimethylformamide by volume in DMF–H_2O solvents. The closed circles (●) are for the experimental data of this paper. The compounds, symbols, and references for other desdimethylaminotetracyclines are: desdimethylaminooxytetracycline, ▲ (2); and desdimethylaminochlortetracycline, ○ (3).

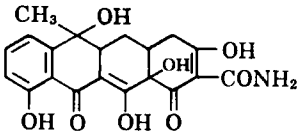
curves of Fig. 1.[1] Linear expressions[2] can be constructed to relate pKa' and %DMF for tetracycline *Ic* as

$$\text{Function A:}\quad pKa' = 0.021\,(\%DMF) + 3.30 \qquad \text{(Eq. 1a)}$$

$$\text{Function B:}\quad pKa' = 0.018\,(\%DMF) + 7.41 \qquad \text{(Eq. 1b)}$$

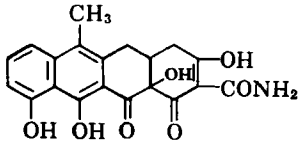
$$\text{Function C:}\quad pKa' = 0.007\,(\%DMF) + 9.62 \qquad \text{(Eq. 1c)}$$

The pKa'-assignments to the labeled functional groups of desdimethylaminotetracycline (hydrogen substituted for the dimethylamino group in *Ic*) are designated by the lettered curves of Fig. 2 (1-4).



II

Desdimethylaminotetracycline



III

Anhydrodesdimethylaminotetracycline

The linear expressions constructed to relate pKa' and %DMF for desdimethylaminotetracycline II are

[1] The assignments used are as published in literature (1–4). However, in light of recent interest in possible reassignment of pKa' values, it is interesting to note that the classical increase of apparent pKa' with decreasing dielectric constant for uncharged acids could argue for the interchange of the assignments of functions B and C. (See Fig. 1.)

[2] Due to a possible change in selective solvation of the dissociating molecule as 0% DMF is approached, the intercept of such expressions may vary slightly from the observed pKa' values in water.

Function A: $pKa' = 0.042\ (\%DMF) + 5.25$ (Eq. 2a)

Function C: $pKa' = 0.023\ (\%DMF) + 8.00$ (Eq. 2c)

Some of the literature data on pKa' (1–4) for the various tetracyclines in %DMF–$\%H_2O$ solutions are also plotted in Figs. 1 and 2 for comparison. It is apparent that the aureomycin and terramycin tetracyclines have lower pKa'-values but a similar relation with %DMF so that Eqs. 1 and 2 may be used as satisfactory estimates of pKa'-variation with %DMF—the same slopes but slightly lower intercepts.

A pKa' of 7.35 in 80%DMF–20%H_2O was observed for 10-benzenesulfonyltetracyclinonitrile. (The nitrile is substituted for the carboxamido group in *Ic*.) This value is consistent with the previously reported pKa' 6.9 at 50%DMF (2,4) for 10-benzenesulfonylterramycinonitrile which was assigned to the dimethylammonium ion. Addition of HCl to 10-benzenesulfonyltetracyclinonitrile showed no titratable group below pH 5 and above pH 2.0. This was consistent with the premise of highly increased acidity of the zwitterionic enolic system with nitril derivatives similar to but more acid than A in I(4).

The anhydrodesdimethylaminotetracycline, III, was observed to have two pKa'-values, pKa_1 = 8.06 (94%DMF), pKa_2 = 10.26 (91%DMF). This compound is analogous to anhydrodesdimethylaminoterramycin previously reported (2).

Now the pKa'-values of anhydroterramycin in water can be given (2) as 3.8 for A, 5.5 for C, and 7.2 for B, where the analogous functionalities are given in I. Since a 1,8-dihydroxybenzophthalide system has a pKa of 4.7 (2), the 5.5 pKa can possibly be assigned to the similar group C in anhydroterramycin; the 7.2 pKa' is consistent with the dimethylamine grouping B, zwitterionic with the carboxamide grouping A.

Removal of the dimethylamino group may consistently decrease the acidity of the carboxamido-enol complex A. It is noted that the pKa' 4.3 is elevated to 7.4, Δ pKa' = 3.1 in 50%DMF, and the pKa' 5.3 is elevated to 9.3, Δ pKa' = 4.0 in 95%DMF, for the change from tetracycline to desdimethylaminotetracycline (Figs. 1 and 2). Using these Δ pKa'-values, it can be predicted that on the insertion of a dimethylamino group into anhydrodesdimethylamino tetracycline III, the now zwitterionic carboxamide-enol complex could be assigned either the pKa'-values of 10.3 − 4.0 = 6.3, or 8.1 − 4.0 = 4.1 at 95%DMF. Because the derived pKa' of 6.3 for 95%DMF is the more consistent of these two possible values with the pKa' of the carboxamido-enol complex of tetracycline in 95%DMF, *i.e.*, 5.4 implies that the pKa_2 = 10.3 (94%DMF) is better assigned to the carboxamido grouping A in III, and the pKa_1 = 8.1 (94%DMF) can be assigned to the dihydroxynapthalene grouping C in III.

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Effect of Zinc Oxide Catalyzed Peroxide on Sterols of Almond, Cottonseed, and Olive Oils

By M. WAFIK GOUDAH and EARL P. GUTH

The phytosterols of almond, cottonseed, and olive oils undergo changes in structure when the oils are irradiated in both ultraviolet and sunlight with zinc oxide. The changes in the phytosterol molecules of the oils indicate a probability that a similar change could occur in the sterols of the skin. These changes might produce a molecule with anti-inflammation activity, thus providing an explanation for the dermatological effect of zinc oxide external preparations.

THE CATALYTIC ACTIVITY of zinc oxide in the photochemical formation of hydrogen peroxide has been confirmed by many workers (1, 2). The pharmaceutical aspects of the phenomenon have also been investigated by Guth, Reese, Mathias, Minardi, Blubaugh, Young, and Lozada (3–8).

Lozada and Guth (8) showed that when zinc oxide-containing ointments were irradiated with ultraviolet light in the presence of water and air the measurable peroxide was less or nonexistant in systems that contained cholesterol or other sterols. This was particulary true when wool fat or hydrophilic petrolatum were components of the ointment base. This lack of peroxide was accounted for by the effect on the sterol molecule.

Infrared spectrographic studies showed that the β-hydroxyl group on cholesterol was oxidized to a carbonyl group. There was also evidence that the double bond shifted from the 5:6 position to the 4:5 position.

With this as a background, a study of the effect of zinc oxide catalyzed peroxide on the sterols in

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olive oil, expressed almond oil, and cottonseed oil was undertaken.

EXPERIMENTAL

Calamine liniment, N.F. IX, was selected as the basic formula for the study. Zinc oxide "Baker" 3.2 Gm. was weighed into 1000-ml. Pyrex beakers, and 100 ml. of 0.2 *M* sodium formate solution was added. The contents were shaken by hand rotation. Ten milliliters of oil was added and the beakers (two at a time) were placed under a Dazor floating fixture ultraviolet lamp, model N. U-58 equipped with a General Electric VA-3 360 watt quartz photo chemical "Ulvirac" lamp.

Metal stirrers driven by Power-Stir model 58 motor, Eberbach Corporation, were used. The stirring was kept on the low speed indicated on the motor and was maintained through a 30-minute period of irradiation. The irradiation unit was completely enclosed within aluminum sheeting.

The phytosterols of each oil were isolated before and after each sample had been irradiated by the following method (9).

The sample of the oil was saponified with 2 *N* alcoholic potassium hydroxide under a reflux condenser. After saponification, most of the alcohol was removed by evaporation. The residual soap was dissolved in hot water and transferred to a separator. The flask was rinsed with hot water, and the rinsing was added to the funnel. After cooling, the unsaponifiable matter was extracted with three successive amounts of ethyl ether. The ethereal solutions were combined and washed with a small quantity of water to remove any residual soap. The ether was removed by distillation, and the residue was dried at 100°.

The unsaponifiable matter was dissolved in ethyl ether, and the solution was transferred to a small porcelain dish. The ether was allowed to evaporate spontaneously, the mass was dried on a water bath and dissolved (after cooling) in the smallest possible quantity of absolute alcohol. The phytosterols were allowed to crystallize from this solution.

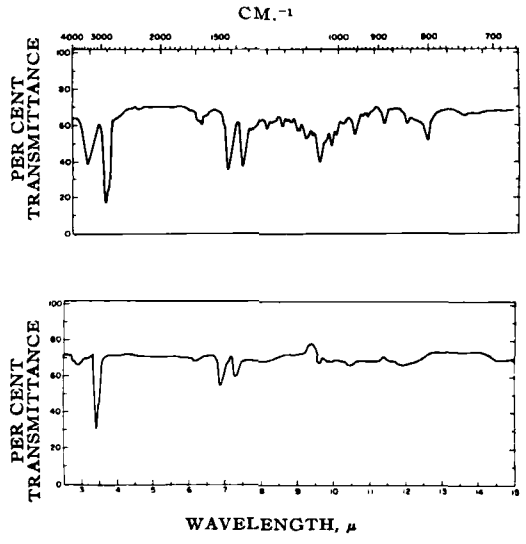

Fig. 1.—Infrared spectra of phytosterols in olive oil (top); in irradiated olive oil (bottom).

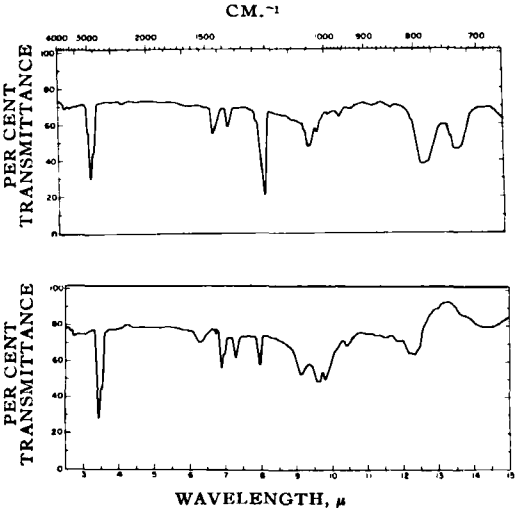

Fig. 2.—Infrared spectra of phytosterols in almond oil (top); in irradiated almond oil (bottom).

The sterols were dissolved in chloroform, and infrared spectrographs were determined in the Perkin–Elmer Infracord (Figs. 1–3). The phytosterols of cottonseed oil were also extracted after a mixture of zinc oxide and the oil had been exposed to the sunlight for 6 hours. The infrared spectra of the extracted phytosterols were determined and are shown in Fig. 3.

RESULTS AND DISCUSSION

The infrared spectra of the phytosterols of the oils isolated before irradiation showed a region from 4000 cm.$^{-1}$ (Figs. 1–3) which is characteristic of the functional groups in the molecule, while the "fingerprint" region is between 1350 cm.$^{-1}$ and 650 cm.$^{-1}$.

Absorption regions between 1055 cm.$^{-1}$ and 950 cm.$^{-1}$ are associated with the hydroxyl group in the sterol molecule. The absorption bands between 1464 cm.$^{-1}$ and 1375 cm.$^{-1}$ are characteristic of methylene group and angular methyl group. The 5:6 double bond gives bands at 840 cm.$^{-1}$ and 800 cm.$^{-1}$ (10). The region between 650 cm.$^{-1}$ and 800 cm.$^{-1}$ is also characteristic of all types of double bonds (11). All other absorption bands are "fingerprints" of the molecule.

The infrared spectra of the phytosterols of olive oil after it has been irradiated showed that variations occurred in the molecule. Bands due to the hydroxyl methyl group and the methylene group, although becoming reduced in intensity, are still present. The "fingerprint" bands showed a notable change. A very pronounced change is indicated by the disappearance of the double bond bands at 800 cm.$^{-1}$ and 840 cm.$^{-1}$.

The infrared spectra of the phytosterols of expressed almond oil after irradiation showed that certain groups disappeared and others shifted. The band at 1200 cm.$^{-1}$ was shifted to 1250 cm.$^{-1}$. As in the case of olive oil, the double bond region between 800 cm.$^{-1}$ and 650 cm.$^{-1}$ disappeared. Bands due to hydroxyl, methyl, and methylene groups were still present.

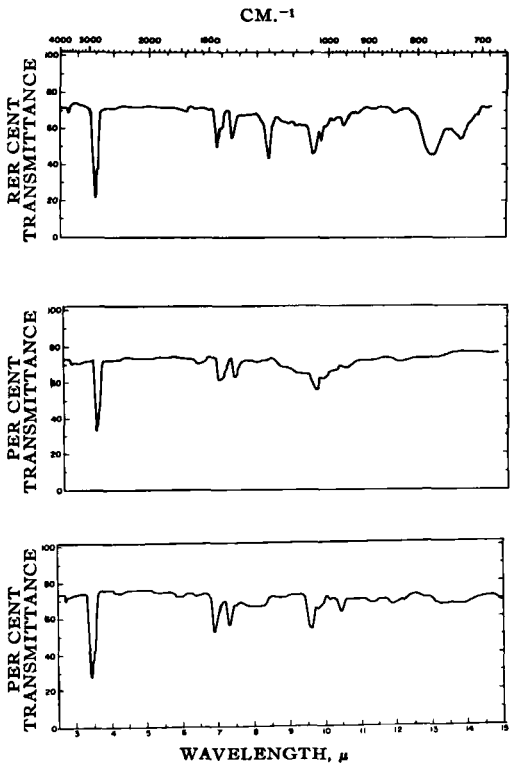

Fig. 3.—Infrared spectra of phytosterols in cottonseed oil (top); in irradiated cottonseed oil (middle); in 6 hour sunlight-exposed cottonseed oil (bottom).

Phytosterols of irradiated cottonseed oil still show bands of the hydroxyl, methyl, and methylene groups but of lessened intensity. The strong bands at 1200 cm.$^{-1}$ disappeared, and great changes in the "fingerprints" occurred. Also, (as with olive oil and almond oil) the double bond region between 800 cm.$^{-1}$ and 650 cm.$^{-1}$ disappeared.

When cottonseed oil, zinc oxide, sodium formate, and water were exposed to sunlight for 6 hours, changes also occurred in the phytosterol molecules. The changes are almost as identical as the changes in the oil irradiated under the ultraviolet lamp with the zinc oxide and sodium formate solution. The hydroxyl band was of greater intensity than when cottonseed oil was irradiated with ultraviolet light.

The disappearance of the double bond region from all the irradiated phytosterols, together with the other changes, indicates beyond doubt that the structure of the phytosterol molecule undergoes changes when exposed to the peroxides produced by zinc oxide and ultraviolet light. In the presence of oxygen–containing compounds such as water and air, Payot (12) found cholesterol susceptible to radiations and oxidizes on and about the 5,6 double bond. On the basis of the infrared spectrum and Payot's findings, it could be said that certain changes occurred in the molecule that might involve oxidation on and about the 5,6 double bond. Since hydrogen peroxide and ultraviolet irradiation energy are both present in this system, a reaction involving radical mechanism initiated by the energy of the ultraviolet very likely occurred in the phytosterol molecule.

When the sterols isolated from the sun-exposed cottonseed oil were tested pharmacologically they showed absence of anti-inflammatory activity. However, the changes which occurred in the molecule could possibly be a step in a chain of reactions which would yield a molecule that has anti-inflammatory effect. Since it has been shown that the production of hydrogen peroxide by zinc oxide and ultraviolet light as well as sunlight can cause structural changes in the sterols of natural lipids, it might be possible that changes could be caused in the sterols of the skin. If this were so, it would be a more rational explanation of the pharmacology of zinc oxide than the statements usually found in standard pharmacological references to zinc oxide action.

CONCLUSIONS

The infrared spectra of the phytosterols before and after irradiation proved that the phytosterols of the oils undergo changes in structure when the latter was irradiated in both ultraviolet and sunlight with zinc oxide. The double bond breakage in the sterol molecule was among the changes that occurred in the molecule.

Sunlight caused the same change in the sterol molecule as did ultraviolet light irradiations. Preliminary pharmacological tests showed that the sterols isolated from the sun-exposed cottonseed oil had no anti-inflammatory activity.

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Colorimetric Determination of Phenylephrine Using 4-Aminoantipyrine

By K. THOMAS KOSHY and H. MITCHNER†

Two colorimetric methods are described for phenylephrine hydrochloride using potassium ferricyanide and 4-aminoantipyrine as reagents. Using 2 per cent sodium borate as the medium, a sufficiently stable color is obtained. This method is suitable for the analysis of phenylephrine in common tablet formulations without prior separation. A second procedure using tris(hydroxymethyl)aminomethane (Tham) buffer, pH 9, and isopropyl alcohol as medium gives a color stable for over 1 hour.

THE COLOR REACTION of 4-aminoantipyrine with phenols has been extensively investigated (1–11). Application of this reagent to the analysis of phenylephrine has been reported by Hiskey and Levin (12). Their procedure consisted of adding to the sample, solutions of potassium ferricyanide, 4-aminoantipyrine, and sodium bicarbonate in the above order and measuring the absorbance 15 minutes after addition of reagents. The waiting period was necessary to allow for the dissipation of an interfering color. The reagent blank had a high initial absorbance which dropped to about 0.06–0.07 (1 cm. cells) after 15 minutes. The color of the sample also faded rapidly during the first 15 minutes and at a less rapid rate thereafter.

Investigation of the reaction of 4-aminoantipyrine and phenylephrine was undertaken because of the specificity of the reaction and the possibility of application in complex mixtures. Critical factors found by literature review and investigation were pH, concentration of reagents, order of addition of reagents, and polarity.

A rapid and convenient procedure was developed utilizing a 2% buffer solution of sodium borate. The interfering red color reported by Hiskey and Levin (12) could be avoided by the addition of 4-aminoantipyrine after dilution of the phenylephrine-potassium ferricyanide reaction mixture with the buffer solution. An additional advantage of the revised procedure was that over a wide range neither the concentration nor the proportion of the two reagents were critical factors. The color obtained was fairly stable, and the absorbance due to the reagent blank was low (Fig. 1). Accurate and reproducible results were obtainable, and the color reaction was found to obey Beer's law in the range of 1–10 mcg./ml.

Studies relative to the increase of the stability of the color indicated that polarity of the medium was a factor. Water-miscible organic solvents were tried in combination with different buffer solutions to improve color stability. It was found that isopropyl alcohol when used with Tham buffer pH 9 had a stabilizing effect. The procedure consisted of the addition of the buffer solution and isopropyl alcohol to the mixture of phenylephrine and potassium ferricyanide, followed by 4-aminoantipyrine and more of the buffer solution to a definite volume. Again, the order of addition of reagents was important, as a more intense but less stable color was obtained if the 4-aminoantipyrine was added prior to the addition of isopropyl alcohol.

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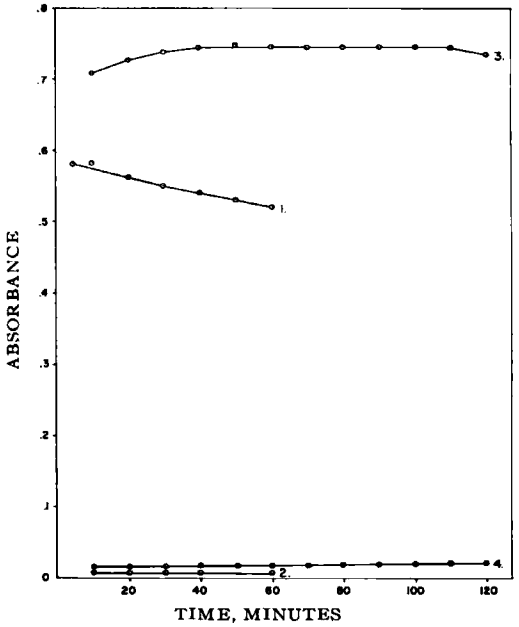

Fig. 1.—Phenylephrine–4-aminoantipyrine color reaction. 1, two per cent sodium borate medium; 2, blank for 1; 3, Tham buffer pH 9 —isopropyl alcohol medium; 4, blank for 3.

The color reaction was reproducible, obeyed Beer's law at concentrations down to 1 mcg./ml., and gave a low blank value. It was 25% more sensitive than the suggested procedure using borate buffer.

Application of the reactions in the two buffer media described was studied with a number of tablet formulations containing phenylephrine. Common tablet excipients and ingredients such as aspirin, ascorbic acid, phenacetin, caffeine, and chlortrimeton did not interfere in the borate buffer system when assaying a filtered aqueous extract of ground tablets for their phenylephrine content. When Tham buffer was utilized, cloudy solutions were frequently encountered on addition of isopropyl alcohol, and this system could not be used without prior separation of phenylephrine from interfering substances.

EXPERIMENTAL

Reagents and Equipment.—4-Aminoantipyrine, Eastman Organic Chemicals, 3% in water[1]; potassium ferricyanide, C.P., 4% in water[1]; sodium

[1] Solution stable for at least 1 week if stored in amber reagent bottle.

borate, $Na_2B_4O_7 \cdot 10H_2O$, A.C.S., Fisher Scientific Co., 2% in water; tris(hydroxymethyl)aminomethane (Tham), purified, Fisher Scientific Co., 50 ml. 0.1 *M* solution adjusted to pH 9 with 0.1 *M* HCl and diluted to 100 ml. with water; phenylephrine hydrochloride, Winthrop Laboratories; Beckman DU spectrophotometer; and 1-cm. Corex cells were utilized.

Procedure Using 2% Sodium Borate Solution.—Three milliliters of an aqueous solution containing 150–450 mcg. of phenylephrine hydrochloride was pipeted into a 50-ml. volumetric flask. One milliliter of potassium ferricyanide reagent was added, and the solution was diluted to about 48 ml. with sodium borate solution. One milliliter of 4-aminoantipyrine reagent was added, and the volume was made up with borate buffer and mixed. The absorbance of this solution was determined immediately at 490 mμ against a reagent blank. The concentration of the sample was calculated by comparison with the color developed simultaneously on a standard solution of phenylephrine hydrochloride.

Procedure Using Tham Buffer and Isopropyl Alcohol.—Three milliliters of an aqueous solution containing approximately 150–450 mcg. of phenylephrine was pipeted into a 50-ml. volumetric flask. One milliliter of potassium ferricyanide was added, followed by 15 ml. of the buffer, 15 ml. of isopropyl alcohol, 1 ml. of 4-aminoantipyrine, and more of the buffer to volume. The contents of the flask were mixed after the addition of each reagent. The absorbance of the solution was determined at 490 mμ against a reagent blank 30 minutes after the development of color. The color developed simultaneously on a standard solution of phenylephrine hydrochloride was used to calculate the concentration of the sample.

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Acute Toxicity of Intravenous Sodium Lauryl Sulfate

By HELMUT F. CASCORBI, FRIEDA G. RUDO, and GORDON G. LU

Sodium lauryl sulfate (SLS) is an excellent emulsifying agent. An emulsion of methoxyflurane in oil, stable to autoclaving, and employing SLS as an emulsifier was prepared. The acute effects of intravenous SLS on red cells, electrocardiogram, vital organs of rabbits, dogs, and monkeys, and isolated hearts of rabbits and frogs were studied. The dose levels were 10 and 50 mg. per cent in 5 per cent glucose or in 3.5 per cent emulsion of methoxyflurane at administration rates of 6.2 ml./Kg./hr. intravenously. The hemolytic effects of SLS and its effect on the electrocardiogram are negligible. SLS evokes a precipitous transient depressor response in dogs. However, SLS has marked acute effect on lungs, kidneys, and especially liver. The hepatotoxicity of SLS seems to preclude its intravenous use in man.

SODIUM LAURYL SULFATE (SLS) has been used in dentifrices for years. Its pharmacologic and toxicologic properties have been studied by a number of investigators (1–5). Recently the Council on Drugs has directed attention to possible hepatosensitivity associated with erythromycin propionate lauryl sulfate (6).

The authors' work with intravenous anesthetic emulsions (7) prompted an investigation of the suitability of SLS as an emulsifier. SLS in concentrations of 2–5 mg. % proved to be an excellent emulsifier and produced a very stable emulsion that tolerated autoclaving for 19 minutes at 15 lb.

After we had established the usefulness of SLS as an emulsifier for emulsions of volatile anesthetics, we then proceeded to investigate its acute toxicity upon intravenous administration.

Received October 15, 1962, from the School of Medicine, Department of Pharmacology, University of Maryland, Baltimore.
Accepted for publication November 13, 1962.

The LD_{50} of SLS upon intravenous administration in rats and mice (kindly supplied to us by K. K. Chen) was found to be 118.2 ± 7.2 mg./Kg.

The properties that were of special interest to us were (*a*) lysis of the red cell membrane, (*b*) its influence on the electric activity of the heart, and (*c*) its acute toxic effects on liver, kidneys, and lungs.

METHODS

Hemolysis.—Two mongrel dogs were anesthetized with 25 mg./Kg. pentobarbital intravenously. The animals were then infused with a solution of 50 mg. % SLS in 5% glucose at a rate of 6.2 ml./Kg./hr. for 1 hour.

Prior to and after infusion, blood samples for hemolysis were taken. Samples were drawn through 17-gauge needles in plastic syringes, bubble free. Plastic centrifuge tubes and a well balanced centrifuge were used to avoid mechanical disrupture of the red cells.

borate, $Na_2B_4O_7 \cdot 10H_2O$, A.C.S., Fisher Scientific Co., 2% in water; tris(hydroxymethyl)aminomethane (Tham), purified, Fisher Scientific Co., 50 ml. 0.1 *M* solution adjusted to pH 9 with 0.1 *M* HCl and diluted to 100 ml. with water; phenylephrine hydrochloride, Winthrop Laboratories; Beckman DU spectrophotometer; and 1-cm. Corex cells were utilized.

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TABLE I.—EFFECT OF SLS ON VITAL ORGANS OF DOGS

Dog No.	SLS, 10 Mg. %	Diagnosis		
		Lungs	Kidneys	Liver
12	Emulsion	Atelectasis, mild	Cloudy swelling	Cloudy swelling
15	Emulsion	Atelectasis pneumonitis	Acute pyelonephritis	Subhepatic necrosis
16	Emulsion	Acute congestion	Normal	Acute congestion
17	Glucose, 5%	Moderate congestion	Mild cloudy swelling	Normal
18	Glucose, 5%	Normal	Moderate cloudy swelling	Severe cloudy swelling. Hemorrhage focal necrosis severe.
19	Glucose, 5%	Normal	Minimal cloudy swelling	Moderate cloudy swelling

TABLE II.—EFFECT OF SLS ON VITAL ORGANS OF RABBITS

Rabbit No.	Lungs	Kidneys	Liver	Bone Marrow	Diagnosis
1 3/1/62	Focal inflammatory reactions throughout specimen; some in bronchi. Much of lung is air containing.	Normal tubules. Cloudy swelling of tubules with moderate glomerular congestion.	Pericentral necrosis of liver cells involving 3/5 of each lobule. Bile ducts, vessels, and triads are normal.	30% reduction with normal ratio of cells. (30% depression of cellulloid.)	Hyperplasia, bone marrow. Necrosis, pericentral; liver severe. Cloudy swelling, kidneys. Globular pneumonia acute.
2 3/1/62	No lesions	Cloudy swelling of tubules. Normal glomeruli.	Mild pericentral necrosis in 3/5 lobule which appears to be reversible. Normal triads and bile ducts.	Normal	Cloudy swelling, kidneys. Fatty necrosis pericentral liver.
3 3/1/62	Collapse and congestion with early inflammation near bronchi; early. Abundant aeration is still present.	Very mild cloudy swelling only with normal glomeruli and tubules.	Considerable pericentral necrosis and congestion involving 50% of each lobule. Bile ducts, triads, and periportal cells not affected.	No Specimen	Necrosis, pericentral; liver severe. Pulmonary congestion. Pneumonia, lobular.

Two additional mongrel dogs were treated in a similar manner, employing a 10 mg. % SLS in 5% glucose solution for infusion.

Electrocardiogram and Blood Pressure of Dogs.—Nine mongrel dogs and two monkeys were anesthetized with pentobarbital sodium (25 mg./Kg. i.v.) and infused in the foregoing manner.

Two dogs were infused with a solution of 50 mg. % SLS in 5% glucose, and seven dogs were infused with a solution of 10 mg. % SLS in 5% glucose for 1 hour each. One monkey was infused with a solution of 15 mg. % SLS in 5% glucose, and another monkey was infused with 20 mg. % SLS in 5% glucose, each for 30 minutes with 6.2 ml./Kg./hr. Lead II of the electrocardiogram was monitored in all animals.

Three mongrel dogs under ether anesthesia were injected intravenously with varying amounts (3.5 mg./Kg. to 7.0 mg./Kg.) of SLS in a concentration of 70 mg. %, and the blood pressure was monitored from the carotid artery.

EKG recordings (Lead II) were obtained at the same time.

Acute Toxic Effects On Liver, Lungs, and Kidneys.—Three mongrel dogs were anesthetized with thiopental sodium, and a control liver biopsy was obtained through a midline incision. Anesthesia was then continued with an emulsion of methoxyflurane (3.5 ml. methoxyflurane, 3.0 ml. cottonseed oil, 1.0 ml. ethanol, 0.5 Gm. Pluronic F 68, 4.2 Gm. glucose and water *q.s. ad.* 100.00 ml.) to which 10 mg. % of SLS had been added after emulsification. The dogs were anesthetized with the emulsion for 3 hours. The dose was determined by the individual susceptibility of the animals to the anesthetic. The dose was 10.8, 9.1, and 6.6 ml./Kg./hr., respectively. After 3 hours of anesthesia, biopsies of liver, lungs, and kidneys were taken, and the animals were sacrificed.

Three more dogs were anesthetized with pentobarbital sodium (25 mg./Kg. i.v. with subsequent doses if necessary), a biopsy of the liver was obtained, and then an infusion of 10 mg. % SLS in 5% glucose administered at the rate of 6.2 ml./Kg./hr. for 3 hours. After this time another biopsy of the liver and biopsies of the lungs and kidneys were obtained, and the animals were sacrificed.

The organ specimens were fixated with 1% $CaCl_2$ in 10% formalin and sent to Dr. John A. Wagner of the Department of Neuropathology for the examination.

Five rabbits were anesthetized daily with 3.5%

emulsion of methoxyflurane by intravenous injection into the marginal ear vein. The emulsion contained 10 mg. % of SLS. Anesthesia was maintained for 30 minutes and repeated five times. The average dose was 16 ml./Kg./hr.

Isolated Rabbit's Heart.—The modified Langendorf procedure was used (8).

Perfused Frogs' Hearts *In Situ.*—The Greene frog-heart perfusion was used.

RESULTS

Hemolysis.—The plasma of three of the dogs was free of any visible hemolysis, whereas the plasma of one dog that received 10 mg. % SLS in 5% glucose showed a trace of hemolysis that was found to be below 30 mg. % hemoglobin.

Electrocardiograms.—The EKG tracings were remarkably devoid of significant findings. We observed peaking of *T* in two cases and an inversion of the *T*-wave in one case. The EKG of the monkeys was not changed.

Blood Pressures.—Dosage levels of SLS up to 3.5 mg./Kg. caused a fall in mean arterial pressure of 7–21% of the norm. Levels of 7 mg./Kg. evoked a 36–40% fall in blood pressure. Electrocardiographic changes were not significant.

Acute Toxic Effects On Liver, Lungs, and Kidneys.—The pathological diagnosis of the six dogs are shown in Table I. The table shows clearly that SLS causes cloudy swelling and congestion in lungs, kidneys, and liver with a preponderance on hepatic damage where even focal necrosis was observed.

Isolated Rabbit's Heart.—SLS dissolved in the perfusion fluid (60 mcg.) had no effect upon the amplitude of contraction of the rabbit's heart in two experiments. Levels of 120 and 240 mcg. caused a moderate depression of contractility.

Levels of 30 and 60 mcg. caused irregular transient coronary flow responses. Levels of 120 and 240 mcg. caused diminution of 35 and 80%, respectively.

Perfused Frogs' Hearts *In Situ.*—In three hearts 1 mg. % of SLS had no effect on the rate or amplitude of contraction. A level of 10 mg. % reduced the rate and amplitude of contraction in two hearts and in the third heart caused cardiac stoppage.

Rabbits.—The results of repeated administration of SLS in emulsion to rabbits are detailed in Table II. Two of the animals died before the study was completed.

DISCUSSION

We administered 10 mg. % SLS in 5% glucose solution to ascertain whether the hepatic toxicity that was found for 10 mg. % SLS in an emulsion could be ascribed to one of the constituents of the emulsion.

The recurrence of renal and especially hepatic changes compel us to ascribe these changes to the action of SLS. However, even at these levels hemolysis was not encountered. We believe that SLS should not be used intravenously in man.

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Modification in Sample Preparation for the Microbiological Assay of Vitamin B_{12}

By ELKO M. STAPERT, HELEN CRAIN, and ROBERT NEEDHAM

A modification of the U.S.P. XVI method for the assay of vitamin B_{12} eliminates some negative bias encountered with some products and permits quantitative recovery of the vitamin. Soft-elastic and hard-filled capsules are blended in the buffered sodium metabisulfite solution before autoclaving. This procedure is particularly necessary to assay older capsules. Products containing reducing sugars are protected with potassium cyanide instead of sodium metabisulfite.

BLENDING WHOLE CAPSULES BEFORE ASSAY

THE MICROBIOLOGICAL METHOD for assaying products containing vitamin B_{12} is described in U.S.P. XVI (1). In this method the product is placed into a buffered solution of sodium metabisulfite and autoclaved for 10 minutes during which all convertible B_{12} is changed to the more stable sulfite form. In assaying many types of products, lower values were obtained with samples stored at room temperature for 18 and 24 months by U.S.P. XVI method than by the assay described in the U.S.P. XIV, third supplement (2).

When whole capsules are placed in the sodium metabisulfite solution, a period of time is required before the contents are in contact with the sodium metabisulfite. A modification in sample preparation described in this paper permits quantitative recovery of the vitamin in contrast to the U.S.P. method where some B_{12} may be destroyed.

Experimental Methods

Sample Preparation with KCN.—All operations and ingredients were as directed in the U.S.P. XVI assay method except the sample preparation.

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emulsion of methoxyflurane by intravenous injection into the marginal ear vein. The emulsion contained 10 mg. % of SLS. Anesthesia was maintained for 30 minutes and repeated five times. The average dose was 16 ml./Kg./hr.

Isolated Rabbit's Heart.—The modified Langendorf procedure was used (8).

Perfused Frogs' Hearts *In Situ.*—The Greene frog-heart perfusion was used.

RESULTS

Hemolysis.—The plasma of three of the dogs was free of any visible hemolysis, whereas the plasma of one dog that received 10 mg. % SLS in 5% glucose showed a trace of hemolysis that was found to be below 30 mg. % hemoglobin.

Electrocardiograms.—The EKG tracings were remarkably devoid of significant findings. We observed peaking of *T* in two cases and an inversion of the *T*-wave in one case. The EKG of the monkeys was not changed.

Blood Pressures.—Dosage levels of SLS up to 3.5 mg./Kg. caused a fall in mean arterial pressure of 7–21% of the norm. Levels of 7 mg./Kg. evoked a 36–40% fall in blood pressure. Electrocardiographic changes were not significant.

Acute Toxic Effects On Liver, Lungs, and Kidneys.—The pathological diagnosis of the six dogs are shown in Table I. The table shows clearly that SLS causes cloudy swelling and congestion in lungs, kidneys, and liver with a preponderance on hepatic damage where even focal necrosis was observed.

Isolated Rabbit's Heart.—SLS dissolved in the perfusion fluid (60 mcg.) had no effect upon the amplitude of contraction of the rabbit's heart in two experiments. Levels of 120 and 240 mcg. caused a moderate depression of contractility.

Levels of 30 and 60 mcg. caused irregular transient coronary flow responses. Levels of 120 and 240 mcg. caused diminution of 35 and 80%, respectively.

Perfused Frogs' Hearts *In Situ.*—In three hearts 1 mg. % of SLS had no effect on the rate or amplitude of contraction. A level of 10 mg. % reduced the rate and amplitude of contraction in two hearts and in the third heart caused cardiac stoppage.

Rabbits.—The results of repeated administration of SLS in emulsion to rabbits are detailed in Table II. Two of the animals died before the study was completed.

DISCUSSION

We administered 10 mg. % SLS in 5% glucose solution to ascertain whether the hepatic toxicity that was found for 10 mg. % SLS in an emulsion could be ascribed to one of the constituents of the emulsion.

The recurrence of renal and especially hepatic changes compel us to ascribe these changes to the action of SLS. However, even at these levels hemolysis was not encountered. We believe that SLS should not be used intravenously in man.

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TABLE I.—PER CENT RECOVERY OF VITAMIN B_{12} FROM FRESH SOFT-ELASTIC CAPSULES WITH VARIOUS PREPARATION METHODS[a]

KCN Method	U.S.P. Method	U.S.P. Method Modified
97.3	92.7	...
97.3	90.0	...
100.5	93.1	...
101.6	87.5	104.2
101.4	88.9	103.5
...	91.4	97.3
...	90.0	105.0
...	92.8	102.9
...	90.0	102.9
...	93.6	98.7
...	92.7	98.7
...	90.0	98.7
...	94.1	100.5
...	93.1	99.9

[a] All volumes were 300 ml. Per cent recoveries are based on theoretical values.

TABLE II.—PER CENT RECOVERY OF VITAMIN B_{12} FROM SOFT-ELASTIC CAPSULES STORED AT ROOM TEMPERATURE[a]

KCN Method	U.S.P. Method		U.S.P. Method Modified
18 Mo.	18 Mo.	24 Mo.	24 Mo.
98	82	79.5	99.7
100.5	87.7	79	101.2
96	84	81	103.5
92	79	...	...

[a] All volumes were 300 ml. Per cent recoveries are based on theoretical values.

Capsules were blended for 2 minutes in 300 ml. of a buffer solution in which 100 mg. of potassium cyanide replaced the sodium metabisulfite. The samples were made to volume and diluted to the desired concentration immediately. The autoclaving step was eliminated.

U.S.P. Assay Method.—All operations were carried out as specified in the U.S.P.

Sample Preparation by U.S.P. Method Modified.—The U.S.P. procedure was carried out as specified except that capsules were blended 2 minutes in a Waring Blendor containing the required amount of sodium metabisulfite solution. Hard-filled capsules were opened and dropped into the sodium metabisulfite solution before autoclaving. This method is similar to the one published after our studies were initiated (3).

Results and Discussion

The assay results obtained with the three methods of sample preparations as applied to both soft-elastic and hard-filled capsules containing 2.2 or 5.5 mcg. vitamin B_{12} per capsule are shown in Tables I, II, and III. The tables include results with freshly made capsules as well as those stored at room temperature. Soft-elastic capsules which had been stored at room temperature for 18 or 24 months showed greater differences than freshly prepared capsules. This was probably due to the gradual conversion of cyanocobalamin to the hydroxo form by reducing substances present in the product. The hydroxo form is less stable to heat and may be destroyed during autoclaving before the capsules are dissolved. Full recoveries were obtained when the capsule fill was immediately in contact with the potassium cyanide or sodium metabisulfite.

Summary

It is apparent from results in the tables that total B_{12} potency can be measured in more products using the modification described than by the U.S.P. XVI method. It is suggested that all products be dissolved in the sodium metabisulfite solution prior to autoclaving.

INACTIVATION OF B_{12} BY REDUCING SUGARS

Results obtained by the U.S.P. microbiological assay for B_{12} have been compared with results using a Co^{60} radioactive trace method. Products containing reducing sugars gave lower values with the microbiological method than with the tracer method; this difference was great enough to interest us in investigating the cause for the lower values. Experiments were set up to determine the effect of various sugars on vitamin B_{12} and its protection by sodium metabisulfite and potassium cyanide in the U.S.P. assay procedure.

Experimental

Crystalline vitamin B_{12} was assayed according to the U.S.P. procedure in the presence of various reducing and nonreducing sugars. Intrinsic factor concentrate N.F. was treated in a similar manner.

Crystalline vitamin B_{12} plus lactose was prepared for assay according to the U.S.P. method, except that 100 mg. of potassium cyanide was added instead of the sodium metabisulfite. The KCN was added to the buffer solution used in preparing the sodium metabisulfite solution to keep the mixture at the proper pH value. Another modification was the elimination of the autoclaving step as a control for determining whether the sugars might have some toxic effect on the organism. Only reducing sugars were used in this modification.

Results and Discussion

The results recorded in Table IV show that the

TABLE III.—PER CENT RECOVERY OF VITAMIN B_{12} FROM HARD-FILLED CAPSULES, NEW STOCK[a]

KCN Method	U.S.P. Method	U.S.P. Method Modified
106	77.8	104
106	89	104
105.2	86	106

[a] All volumes were 300 ml. Per cent recoveries are based on theoretical values.

TABLE IV.—EFFECT OF SUGARS ON VITAMIN B_{12} IN THE U.S.P. MICROBIOLOGICAL ASSAY[a]

Sugars, 2 Gm. + 50 mcg. B_{12}	Assay Method	Rec., %[b]
None	U.S.P.	100
Dextrose	U.S.P.	61.2
Galactose	U.S.P.	61.7
Mannose	U.S.P.	72.6
Arabinose	U.S.P.	61.3
Lactose	U.S.P.	65.7
Lactose	U.S.P.[c]	101.5
Sucrose	U.S.P.	100.3
Mannitol	U.S.P.	96.4
None	U.S.P. No $Na_2S_2O_5$ or KCN	84.0–82.7–90.8

[a] All volumes were 300 ml. [b] Per cent recoveries are based on theoretical values. [c] KCN replacing $Na_2S_2O_5$.

TABLE V.—EFFECT OF LACTOSE VITAMIN B_{12} AND B_{12} WITH INTRINSIC FACTOR CONCENTRATE (IFC) IN THE U.S.P. MICROBIOLOGICAL ASSAY[a]

B_{12}, Mcg.	Lactose	Rec., %[b]	Mcg. B_{12} as IFC	Lactose	Rec., %[b]
50	0	100.8–000	50	0	96–00
50	1	78.5–79	50	1	83.5–86.5
50	2	65.5–68.7	50	2	80.5–80.3
50	3	59.0–70.0	50	3	80.4–75.0
50	5	59.0–66.6	50	5	57.3–71.5

[a] All volumes were 300 ml. [b] Per cent recoveries are based on theoretical values.

TABLE VI.—EFFECT OF SUGARS ON VITAMIN B_{12} BY A MODIFIED ASSAY METHOD[a]

B_{12}, Mcg.	Sugars, 2 Gm.	Rec., %[b]
50	Lactose	99
50	Dextrose	100
50	Galactose	100.3
50	Mannose	100.4

[a] Samples made up without sodium metabisulfite and not autoclaved. [b] Per cent recoveries are based on theoretical values.

five reducing sugars affect the recovery of vitamin B_{12} to approximately the same amount. Full recovery of the B_{12} could be obtained in the presence of lactose when protected by potassium cyanide instead of sodium metabisulfite. Recoveries were complete in the presence of nonreducing sugars. It will also be noted that a sample of vitamin B_{12} protected by neither potassium cyanide nor sodium metabisulfite but autoclaved in the presence of water only gave a much higher value than the samples treated with reducing sugars and protected with sodium metabisulfite. This is further evidence that reducing sugars destroy vitamin B_{12} in the autoclaving step. The results indicating instability of vitamin B_{12} in the presence of reducing sugars confirm the report by Barr, Kohn, and Tice (4).

Table V shows the effect of increasing amounts of lactose in the assay of B_{12} and B_{12} with intrinsic factor concentrate. As indicated in the table, there is progressively more destruction of the vitamin with increasing amounts of lactose. This would probably be true with all reducing sugars. It appears that there may be some protection of the vitamin when B_{12} is combined with intrinsic factor concentrate, but the destruction is still progressive with increasing amounts of lactose.

Table VI shows the effect of reducing sugars when the samples are not subjected to the autoclaving step and not protected with sodium metabisulfite. This was a control to determine whether these sugars might have some affect on the growth of the test organism. As can be seen in the table, full recovery of the vitamin is obtained.

Some studies were made concerning the effect of dextrose in the medium during incubation of *Lactobacillus leichmanii* in the vitamin B_{12} assay. Since dextrose tends to reduce B_{12}, it should show its effect during the incubation period. In this experiment, which is shown in Fig. 1, a 15% greater growth was obtained with the sucrose medium. The authors' interpretation is that less vitamin is available to the test organism and therefore a depressed growth response is obtained. This may not affect assay precision since presumably all standard and test samples would be affected alike by the presence of dextrose in the medium.

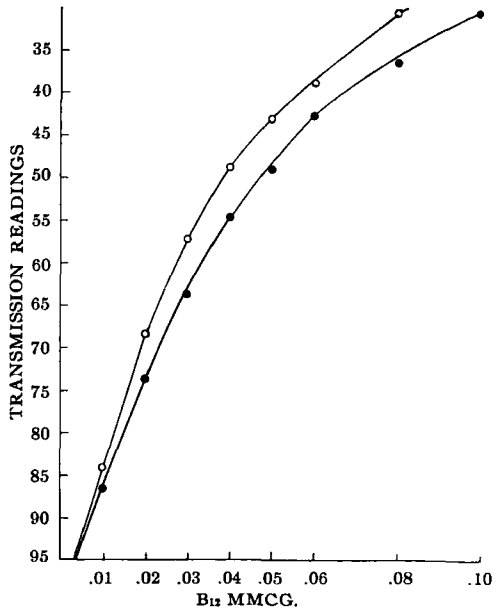

Fig. 1.—Effect of dextrose on vitamin B_{12} during incubation. ○, 40 Gm. sucrose per L. of medium; ●, 40 Gm. dextrose per L. of medium.

Summary

The U.S.P. XVI microbiological assay for vitamin B_{12} has been modified by replacing the sodium metabisulfite with potassium cyanide. This modification permits better recovery of vitamin B_{12} on products containing reducing sugars. The data indicate that potassium cyanide gives better protection in the presence of lactose during autoclaving than sodium metabisulfite.

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Fluorometric Assay of Yohimbine

By H. C. CHIANG and W. F. CHEN

The fluorescence of solutions of yohimbine under ultraviolet light is increased by heating with hydrogen peroxide and is quantitatively related to its concentration. An assay procedure has been developed for pharmaceutical preparations containing yohimbine.

YOHIMBINE has been used medicinally as an aphrodisiac, but there is no convincing evidence for such an effect (1). However, in Formosa it is often illegally mixed in male hormone preparations. Consequently, there is an urgent need to find a suitable method for determining yohimbine in such preparations.

Previous analytical methods have been based on the color reactions of yohimbine with Reinecke salt (2), *p*-dimethylaminobenzaldehyde (3), vanillin (4), and xanthydrol (5). But the interference of hormones as well as vitamins make the above methods unsuitable for the determination of yohimbine in these combinations.

Yohimbine and other rauwolfia alkaloids have fluorescence under ultraviolet light, and this property has been used to determine their location on chromatograms (6–8).

We have found that the intensity of the fluorescence of yohimbine solutions heated with hydrogen peroxide is proportional to the concentration. This procedure has proved to be effective in determining yohimbine in preparations containing methyltestosterone, vitamin B_1, vitamin B_2, *dl*-methionine, and strychnine nitrate.

EXPERIMENTAL

Reagent.—Standard yohimbine solution containing 20 mcg. per ml. in 5 *N* acetic acid.

Apparatus.—Kotaki ultramicro fluorophotometer with 48 × 40 × 8 mm. cells.

Reaction.—It was found that a suitable fluorescence could be obtained by adding 1.0 ml. of 3% hydrogen peroxide to the solution and heating the tube in a boiling water bath for about 45 minutes. The fluorescence developed in this manner showed no change on standing 10 hours. Also the concentration of yohimbine to fluorometric reading follows a straight line relationship. The results of these experiments are summarized in Fig. 1.

Determination of Yohimbine in Hormone-Vitamin Preparations.—Transfer a portion of powder equivalent to 10 mg. of yohimbine-HCl to a 100-ml. volumetric flask. Add 2% tartaric acid solution to the mark, and shake the mixture occasionally during 2 hours; filter, if necessary, rejecting the first 20 ml. of filtrate. Pipet 20 ml. of filtrate into a separator. Then add ammonia T. S. to distinct alkalinity, and extract yohimbine with 20, 20, 10, 10, and 10-ml. quantities of ether. Wash the combined ether extracts with two 10-ml. portions of water. Extract the washings with 10 ml. of ether; add this ether to the combined ether extracts and evaporate the ether on a steam bath to dryness. Dissolve the residue in 100 ml. of 5 *N* acetic acid to make a solution of concentration of 20 mcg./ml.

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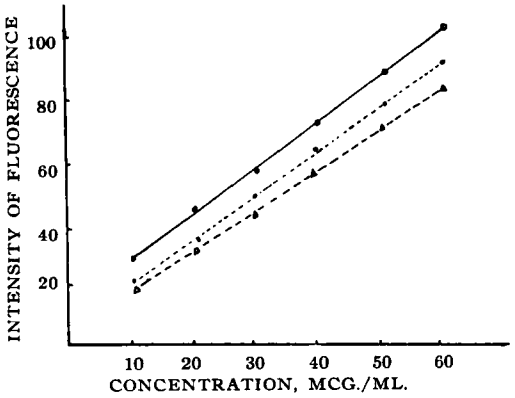

Fig. 1.—Relationship between heating time, concentration, and intensity of fluorescence. Key: O——O——O, 45–60 minutes of heating time; ● . . . . . ● . . . . . ●, 15 minutes of heating time; △ – – – △ – – – △, 5 minutes of heating time.

TABLE I.—SAMPLE PRESCRIPTIONS AND RESULTS OF EXPERIMENTS

Description	Capsule 1, mg.	Capsule 2, mg.	Capsule 3, mg.	Mixture 1, mg.
Methyltestosterone	10	10	10	10
Vitamin B_1	5	10	10	10
Vitamin B_2	2.5	...	...	2.5
dl-Methionine	35	...	...	30
Strychnine nitrate	...	1	...	1
Amino ethane sulfonic acid	...	...	30	30
Calcium glycerophosphate	...	...	200	200
Yohimbine-HCl	2	2	2	0
Av. recovery, %	100.1 ± 1.8	101.6 ± 1.4	99.4 ± 0.2	0

Accurately pipet 2.0 ml. of this solution and standard solution respectively into a 10-ml. volumetric flask. Add 1 ml. of 3% hydrogen peroxide to each, dilute to exact volume with 5 *N* acetic acid, and mix well. Heat in boiling water for 45 minutes. Then cool to room temperature and measure the fluorescence. The concentration of yohimbine hydrochloride in the sample solution is calculated

$$\frac{\text{Galvanometer reading of sample}}{\text{Galvanometer reading of standard}} \times \text{mcg. of yohimbine HCl in standard solution} = \text{mcg. of yohimbine HCl in sample solution.}$$

RESULTS AND DISCUSSION

The proposed method of assay is applied to the mixtures of known yohimbine content. Sample prescriptions and results of experiments are presented in Table I. The results have been shown to be free of interference from most of the vitamins and substances frequently associated with yohimbine.

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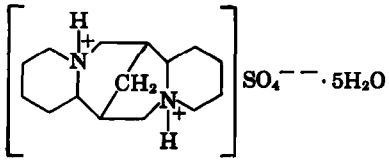
Quantitative Determination of Sparteine Sulfate

By PAUL TURI and DAN GROSSMAN

Official compendia fail to include assay methods for pharmaceutical preparations of sparteine sulfate. A direct acidimetric titration method for sparteine sulfate and an indirect titration of the sparteine base, obtained by distillation from a solution of sparteine sulfate, were investigated in a comparative study. The accuracy and applicability of both methods are evaluated.

SPARTEINE, an alkaloid of *Cytisus scoparius* L. (syn. *Sarothamnus scoparius*, Koch), *Lupinus luteus* L. and other plants of the *Leguminosae*, has been known as a medicinal agent for more than 70 years (1).



Sparteine had been formerly recommended in tachycardia, functional palpitation of the heart, and as a diuretic agent.

"The National Formulary," 8th ed., 1946, monograph (2) for sparteine sulfate (the last official U. S. compendium to list this drug) fails to include a method of assay. Recent interest in sparteine sulfate as an adjunct in induction of labor (3) prompted an evaluation of the known quantitative assay procedures.

In a survey of several suggested methods Higuchi and Bodin (4) list four methods for the determination of sparteine sulfate: (*a*) a colorimetric assay through the Reineckate salt, (*b*) an acid dye procedure (assaying colorimetrically a chloroform extract of the compound formed with acid methyl orange), (*c*) a polarographic method, and (*d*) a gravimetric determination through the silicotungstate salt.

The official method in the "Pharmacopoea Helvetica V" (5) for sparteine sulfate assay is a direct acidimetric titration. This procedure is based on the principle that mineral acid salts of very weak nitrogen bases in aqueous solution can be titrated directly with strong alkaline titrants to the phenolphthalein red end point, since the liberated weak base does not interfere with the indicator (6).

Our objective was to select a simple and rapid method with satisfactory accuracy. The direct titration method requires only a single step; therefore its advantages are obvious. To increase the specificity of the analysis, we adapted another titration technique which included a separation step. The principle of distillation of the alkaloid base and back titration of volatile alkaloids was utilized.

The scope of this study is a comparison between the two acidimetric titration methods: *method I*, sparteine sulfate is titrated directly with 0.1 *N* sodium hydroxide solution; and *method II*, sparteine sulfate salt is converted to the base, distilled with steam into a measured volume of acid, and the excess acid is back-titrated.

EXPERIMENTAL

Reagents and Test Solutions

Sparteine sulfate: a commercial sample of sparteine sulfate, N.F. VIII, was used in this study without further purification m.p. 136° dec., $[\alpha]_D^{25} = -21.5°$ (c = 4 in water); 0.1 *N* NaOH; 0.1 *N* HCl; methyl orange T.S.; phenolphthalein T.S.; hydrogen peroxide T.S.; 0.1 *N* H_2SO_4; sulfuric acid, diluted; 0.1 *N* $KMnO_4$; 50% NaOH: 10 Gm. of NaOH (U.S.P. XVI) dissolved in 10 ml. of water. Volumetric and test solutions meet U.S.P. XVI specifications.

Analytical Procedures

Sample A.—Approximately 1.0 Gm. of sparteine sulfate ($C_{15}H_{26}N_2 \cdot H_2SO_4 \cdot 5H_2O$, mol. wt. 422.53), accurately weighed, was dissolved in water in a 25-ml. volumetric flask, and the volume was adjusted to mark at 20° (stock solution).

Method I: 5.0 ml. of the stock solution was transferred into an Erlenmeyer flask, one drop of

TABLE I.—COMPARATIVE ASSAY RESULTS FOR SPARTEINE SULFATE

Sample No.	Sample Type	Method I %	Method I d_a	Method II %	Method II d_a
1	A	100.43	0.13	99.68	−0.60
2	A	100.43	0.13	98.84	−1.44
3	A	100.35	0.05	100.88	0.60
4	B	101.00	0.70	101.50	1.22
5	B	100.00	−0.30	102.50	2.22
6	B	99.00	−1.30	100.00	−0.28
7	B	99.50	−0.80	101.50	1.22
8	B	100.50	0.20	99.00	−1.28
9	B	101.50	1.20	98.60	−1.68

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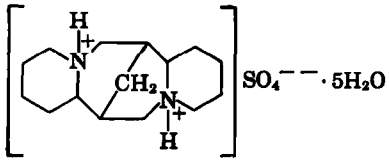
Quantitative Determination of Sparteine Sulfate

By PAUL TURI and DAN GROSSMAN

Official compendia fail to include assay methods for pharmaceutical preparations of sparteine sulfate. A direct acidimetric titration method for sparteine sulfate and an indirect titration of the sparteine base, obtained by distillation from a solution of sparteine sulfate, were investigated in a comparative study. The accuracy and applicability of both methods are evaluated.

SPARTEINE, an alkaloid of *Cytisus scoparius* L. (syn. *Sarothamnus scoparius*, Koch), *Lupinus luteus* L. and other plants of the *Leguminosae*, has been known as a medicinal agent for more than 70 years (1).



Sparteine had been formerly recommended in tachycardia, functional palpitation of the heart, and as a diuretic agent.

"The National Formulary," 8th ed., 1946, monograph (2) for sparteine sulfate (the last official U. S. compendium to list this drug) fails to include a method of assay. Recent interest in sparteine sulfate as an adjunct in induction of labor (3) prompted an evaluation of the known quantitative assay procedures.

In a survey of several suggested methods Higuchi and Bodin (4) list four methods for the determination of sparteine sulfate: (*a*) a colorimetric assay through the Reineckate salt, (*b*) an acid dye procedure (assaying colorimetrically a chloroform extract of the compound formed with acid methyl orange), (*c*) a polarographic method, and (*d*) a gravimetric determination through the silicotungstate salt.

The official method in the "Pharmacopoea Helvetica V" (5) for sparteine sulfate assay is a direct acidimetric titration. This procedure is based on the principle that mineral acid salts of very weak nitrogen bases in aqueous solution can be titrated directly with strong alkaline titrants to the phenolphthalein red end point, since the liberated weak base does not interfere with the indicator (6).

Our objective was to select a simple and rapid method with satisfactory accuracy. The direct titration method requires only a single step; therefore its advantages are obvious. To increase the specificity of the analysis, we adapted another titration technique which included a separation step. The principle of distillation of the alkaloid base and back titration of volatile alkaloids was utilized.

The scope of this study is a comparison between the two acidimetric titration methods: *method I*, sparteine sulfate is titrated directly with 0.1 *N* sodium hydroxide solution; and *method II*, sparteine sulfate salt is converted to the base, distilled with steam into a measured volume of acid, and the excess acid is back-titrated.

EXPERIMENTAL

Reagents and Test Solutions

Sparteine sulfate: a commercial sample of sparteine sulfate, N.F. VIII, was used in this study without further purification m.p. 136° dec., $[\alpha]_D^{25} = -21.5°$ (c = 4 in water); 0.1 *N* NaOH; 0.1 *N* HCl; methyl orange T.S.; phenolphthalein T.S.; hydrogen peroxide T.S.; 0.1 *N* H_2SO_4; sulfuric acid, diluted; 0.1 *N* $KMnO_4$; 50% NaOH: 10 Gm. of NaOH (U.S.P. XVI) dissolved in 10 ml. of water. Volumetric and test solutions meet U.S.P. XVI specifications.

Analytical Procedures

Sample A.—Approximately 1.0 Gm. of sparteine sulfate ($C_{15}H_{26}N_2 \cdot H_2SO_4 \cdot 5H_2O$, mol. wt. 422.53), accurately weighed, was dissolved in water in a 25-ml. volumetric flask, and the volume was adjusted to mark at 20° (stock solution).

Method I: 5.0 ml. of the stock solution was transferred into an Erlenmeyer flask, one drop of

TABLE I.—COMPARATIVE ASSAY RESULTS FOR SPARTEINE SULFATE

Sample No.	Sample Type	Method I %	Method I d_a	Method II %	Method II d_a
1	A	100.43	0.13	99.68	−0.60
2	A	100.43	0.13	98.84	−1.44
3	A	100.35	0.05	100.88	0.60
4	B	101.00	0.70	101.50	1.22
5	B	100.00	−0.30	102.50	2.22
6	B	99.00	−1.30	100.00	−0.28
7	B	99.50	−0.80	101.50	1.22
8	B	100.50	0.20	99.00	−1.28
9	B	101.50	1.20	98.60	−1.68

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TABLE II.—STATISTICAL DATA OF SPARTEINE SULFATE ASSAYS

	Method I	Method II
Average Assay Result, %	100.30	100.28
Probable Error,[a] %	0.50	0.93

[a] Probable error was calculated from the following formula 7)

$$r = 0.6745 \sqrt{\frac{\Sigma d a^2}{n - 1}}$$ (n = number of assays)

TABLE III.—ASSAY RESULTS FOR PEROXIDE-TREATED SPARTEINE SULFATE SOLUTIONS

Sample No.	Sample Type	Method I, %	Method II, %
1	C	100.14	90.08
2	C	98.66	89.05

phenolphthalein T.S. was added, and the solution was titrated with 0.1 *N* NaOH to the appearance of the red color. 1 ml. 0.1 *N* NaOH = 42.253 mg. $C_{15}H_{26}N_2 \cdot H_2SO_4 \cdot 5H_2O$.

Method II: 5.0 ml. of the stock solution was transferred into a 300-ml. Kjeldahl flask. A few glass beads, 75 ml. water, and 2 ml. of 50% NaOH were added, and the flask was connected immediately to a condenser fitted with a delivery tube extending below the surface of 15.0 ml. 0.1 *N* HCl in a receiver. Two drops of methyl orange T.S. was added to the receiver as the indicator.[1] About 65 ml. of the distillate was collected in the receiver, followed by a residual titration of the excess acid with 0.1 *N* NaOH. Each ml. of 0.1 *N* HCl is equivalent to 21.1265 mg. $C_{15}H_{26}N_2 \cdot H_2SO_4 \cdot 5H_2O$.

Sample B.—An ampul solution of sparteine sulfate (containing 150 mg. of $C_{15}H_{26}N_2 \cdot H_2SO_4 \cdot 5H_2O$ and 4.5 mg. sodium chloride in 1.0 ml. of water) has been assayed by the two methods in a similar manner to that described above. Experimental results are summarized in Tables I and II.

Degradation Studies

To investigate the effects of degradation products on the two titration methods, the following experiment was performed.

Sample C.—4.0 Gm. sparteine sulfate was dissolved in about 80 ml. of distilled water. Seven milliliters of hydrogen peroxide T.S. and exactly 3.0 ml. of 0.1 *N* H_2SO_4 were added, and this colorless mixture was boiled for 1 hour. The solution was cooled to room temperature. A definite yellow coloration indicated presence of degradation products. Following the addition of exactly 3.0 ml. of 0.1 *N* NaOH, the solution was quantitatively transferred into a 100-ml. volumetric flask and brought up with water to volume (at 20°).[2] Five-milliliter aliquots of the peroxide-treated sparteine sulfate solution was assayed by both methods described above. Assay results are reported in Table III.

RESULTS AND DISCUSSION

The assay series performed on sparteine sulfate solutions (*Samples A* and *B*) facilitated an evaluation of the reproducibility and accuracy of the investigated methods.

As shown in Tables I and II, the averages of nine parallel determinations are very close; the difference of the average percentages is only 0.02%. The probable error is less than 1% for both methods, and only 0.5% for the direct titration procedure (*method I.*)

Solutions of sparteine sulfate exposed to high temperatures (100–160°) for a prolonged period showed insignificant degradation or no degradation. Earlier reported experiments (8) indicate a number of different oxidation products which could be formed by treating sparteine with a variety of oxidizing agents. Our results, reported in Table III, indicate that under the described experimental conditions degradation products develop which do not affect the direct titration method. By the distillation technique (*method II*), an anticipated decrease in alkaloid content appears.

CONCLUSIONS

Our experimental data led us to conclude that the method of choice for pure sparteine sulfate substance or for pharmaceutical preparations containing no interferring ingredients is the direct titration method. In the presence of interferring substances (incorporated in the pharmaceutical formula or developed by degradation), the distillation technique offers the advantage of separating the sparteine base from the nonvolatile ingredients and can be used for quantitative determinations with satisfactory accuracy.

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[1] More recent work indicates that a sharper end point can be obtained by using four drops of bromocresol green-methyl orange mixed indicator (0.2% BCG + 0.02% MO) to the appearance of a light green color.

[2] A 5-ml. aliquot of this solution was acidified with 1 ml. of diluted sulfuric acid, and one drop of 0.1 *N* $KMnO_4$ solution added. A slight pink color indicated absence of hydrogen peroxide.

Modified Automated Apparatus for Determination of Dissolution Rates of Capsules and Tablets

By LOUIS C. SCHROETER and WILLIAM E. HAMLIN

Intermittent filtration and sampling of the dissolution fluid permits the routine use of short light path (1 mm.) spectrophotometer flow cells in the monitoring of the *in vitro* dissolution process of tablets and capsules by an automated method.

CONTINUOUS MONITORING of the dissolution process of a tablet or capsule may be accomplished most easily by cycling a sample of dissolution fluid through a spectrophotometer flow cell and recording the change in absorbance as a function of time (1). Spectrophotometer flow cells of minimum volume ($\leq$ 5 ml.) with light paths from 10–100 mm. may be used to extend the useful range of the continuous cycling method to those dosage forms which contain small amounts of drug(s) with low molar absorptivities. Determination of the dissolution characteristics of tablets containing large amounts of drug(s) with high molar absorptivities through the use of short light path ($\leq$ 5 mm.) flow cells has in the past involved many operational difficulties: particles of insoluble tablet additives tend to collect in the flow cells and continuous filtration of the sample stream results in rapid and progressive diminution of flow rate as filters become clogged with insoluble materials.

This brief report describes a modification of the continuous cycling procedure for automated dissolution rate studies (1) which greatly extends the utility of the method by employing a 1-mm. light-path flow cell. A three-way solenoid valve is used to shunt the circulating sample stream away from the filter, except during the time actual recording of the dissolution process is underway.

EXPERIMENTAL

Equipment.—The arrangement of components for modified continuous cycling with intermittent filtration and sampling is shown in Fig. 1. The U.S.P. disintegration apparatus and basket rack assembly (2) were used to provide reproducible agitation; disks were *not* used. Sampling lines were of shortest possible length and of minimum internal diameter consistent with sampling-line flow rates of 1 to 1.5 ml. per second. Typical lengths of connecting tubing and volumes of components shown in Fig. 1 were as follows: *A*, 70 cm.; *B*, 50 cm.; *C*, 20 cm.; *D*, 10 cm.; *E*, 30 cm.; *F*, 85 cm.; valve (sample port open), 1 ml.; filter, 4.6 ml.; 1 mm. flow cell,[1] 1.0 ml. Configuration of the glass filtering device and the solenoid valve is shown in Fig. 2. An electronic repeat cycle timer[2] was used to actuate the solenoid valve to divert the rapidly circulating sample fluid through the filtering device and flow cell every 1 or 2 minutes for a period of 15–20 seconds. A more detailed description of the automated dissolution rate apparatus and its design requirements has been reported (1).

Procedure.—Operationally, the dissolution rate determination of tablets or capsules by the intermittent filtration-sampling method is the same as that used with the previously reported continuous monitoring process (1). Recorder response was adjusted to read 100 units or 100% drug in solution when a filtered sample stream from the beaker containing drug from one tablet wholly dissolved (100% drug in solution) was pumped through the flow cell. The sampling system was flushed with fresh buffer and connected with a new beaker containing 750 ml. of fresh buffer maintained at 37°. One tablet was placed into the U.S.P. disintegration basket assembly (no disks), immersed into the buffer solution, and agitated at the prescribed rate of 30 cycles per minute. Introduction of the tablet into the buffer marked zero time for the process. The timer was adjusted to divert the sample stream through the filter and flow cell every minute for a period of 15 seconds.

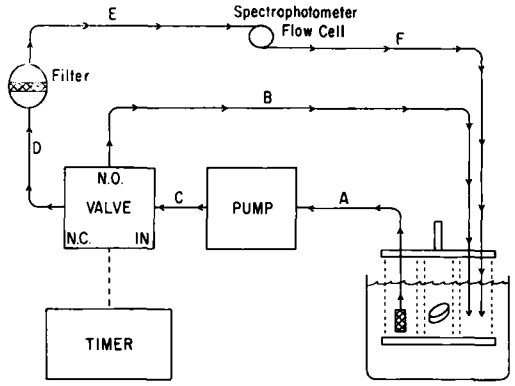

Fig. 1.—Arrangement of components for modified continuous cycling with intermittent filtration and sampling.

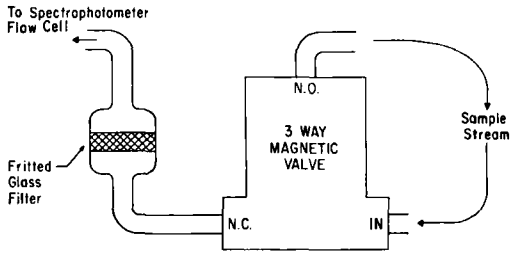

Fig. 2.—Configuration of filter and three-way solenoid valve for intermittent filtration and sampling. The abbreviations "N.O." and "N.C." designate the normally open and normally closed valve parts.

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The excellent technical assistance of N. K. Hine is gratefully acknowledged.

[1] Suprasil flow cell, No. L134FT, 1-mm. light path, Lux Scientific Instrument Corp., New York 13, N. Y.

[2] Repeat cycle timer, model 1, G. C. Wilson and Co., Huntington, W. Va.

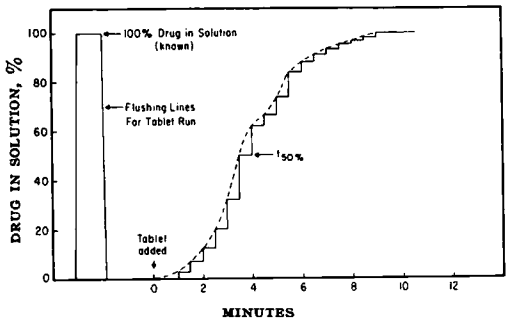

Fig. 3.—Idealized recorder curve obtained with modified continuous cycling method. Actual curves recorded at sufficiently fast chart speeds will show the vertical lines to be more nearly sigmoidal, due to the time lag involved in the establishment of optical density equilibrium. Dashed line indicates dissolution profile; vertical line indicates flow cell open; horizontal line indicates flow cell closed.

RESULTS AND DISCUSSION

Vertical lines shown in the idealized recorder tracing in Fig. 3 represent the rapid change in recorder response which occurs when the sample stream is diverted through the flow cell at 1-minute intervals. Shortly after establishment of equilibrium in the lines, the rapidly flowing sample stream is once again shunted away from the filter and flow cell. During this time no sample flows through the cell; hence a horizontal trace is made by the pen until the valve once again opens to divert the sample stream through the filter and flow cell. Intersection of the horizontal and vertical lines represents a concentration of drug in solution at that time; the dissolution profile of the dosage form is shown by a dashed line joining these intersections or concentration-time values.

Accuracy and reliability of the modified continuous cycling method has been determined by simultaneous independent assays of the dissolution fluid during the dissolution process of various tablets. The dissolution profile of a sulfonylurea tablet in pH 7.2 buffer at 37° was determined in a series of studies conducted on different days by different investigators using the modified method described above and by simultaneous independent spectrophotometric assays of the dissolution fluid. Figure 4 is typical of the excellent agreement which exists between the automated and manual methods.

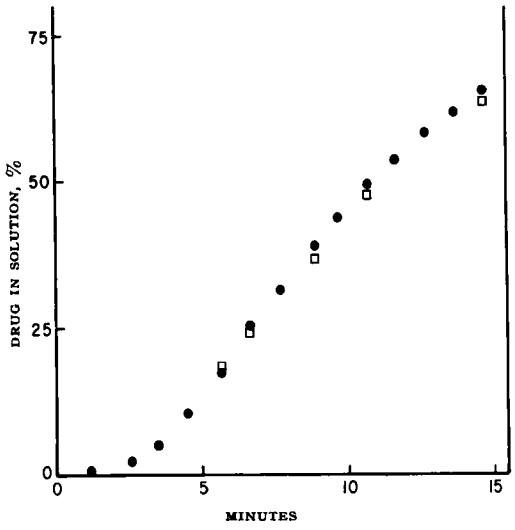

Fig. 4.—Dissolution profile of a sulfonylurea tablet obtained by the automated procedure and by simultaneous independent assay: ●, automated; □, independent assay.

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Ferguson Principle and the Critical Micelle Concentration

By BERNARD ECANOW and FREDERICK P. SIEGEL

The thermodynamic activity values of a series of quaternary ammonium salts were calculated through the use of critical micelle concentrations. The correlation of these thermodynamic activities to the published values of their bactericidal activities is shown through application of the Ferguson principle. The value of the Ferguson principle in helping to point out the possible presence of different mechanisms of activity within a series of similar compounds has been suggested.

FERGUSON (1) has suggested that the toxicities of physically toxic substances should not be compared by the values of the toxic concentrations in the external solution but by the chemical potentials in this phase, which must be identical with the chemical potential at the site of activity. Ferguson used the activity function of G.N. Lewis as the chemical potential. From a review of published data, he showed that "though diverse chemical compounds exert the same toxic effect on a given organism at widely different concentrations, the activities corresponding to these concentrations lie within a relatively narrow range. The differences in activity within this range are ascribed to the effect of chemical constitution."

It has been shown (1, 2) that when the toxic agent is applied as a vapor, the activity is given with "useful" accuracy by the ratio of the partial pressure of the agent over that of the saturated vapor pressure of the substance at the temperature

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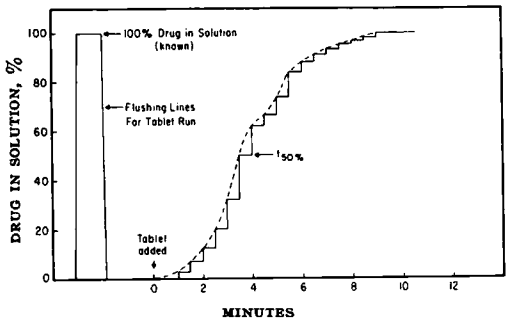

Fig. 3.—Idealized recorder curve obtained with modified continuous cycling method. Actual curves recorded at sufficiently fast chart speeds will show the vertical lines to be more nearly sigmoidal, due to the time lag involved in the establishment of optical density equilibrium. Dashed line indicates dissolution profile; vertical line indicates flow cell open; horizontal line indicates flow cell closed.

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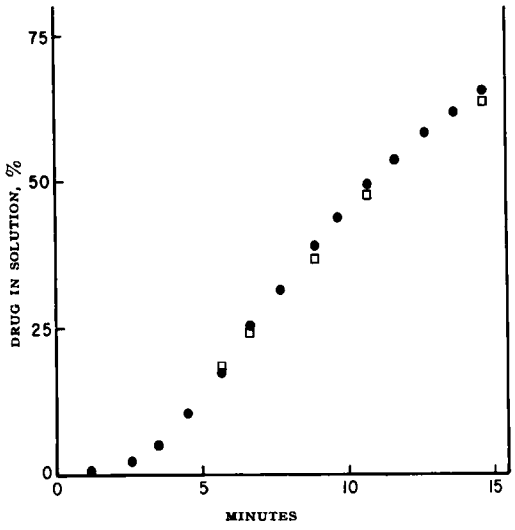

Fig. 4.—Dissolution profile of a sulfonylurea tablet obtained by the automated procedure and by simultaneous independent assay: ●, automated; □, independent assay.

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Ferguson Principle and the Critical Micelle Concentration

By BERNARD ECANOW and FREDERICK P. SIEGEL

The thermodynamic activity values of a series of quaternary ammonium salts were calculated through the use of critical micelle concentrations. The correlation of these thermodynamic activities to the published values of their bactericidal activities is shown through application of the Ferguson principle. The value of the Ferguson principle in helping to point out the possible presence of different mechanisms of activity within a series of similar compounds has been suggested.

FERGUSON (1) has suggested that the toxicities of physically toxic substances should not be compared by the values of the toxic concentrations in the external solution but by the chemical potentials in this phase, which must be identical with the chemical potential at the site of activity. Ferguson used the activity function of G.N. Lewis as the chemical potential. From a review of published data, he showed that "though diverse chemical compounds exert the same toxic effect on a given organism at widely different concentrations, the activities corresponding to these concentrations lie within a relatively narrow range. The differences in activity within this range are ascribed to the effect of chemical constitution."

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TABLE I.—ACTIVITY OF QUARTERNARY AMMONIUM COMPOUNDS

Quaternary Ammonium Chloride	CMC (N)	*M. aureus* Test Concn. $M \times 10^4$	*M. aureus* Killing Time[a], Min.	*E. coli* Test Concn. $M \times 10^4$	*E. coli* Killing Time[a], Min.	Activity *M. aureus*	Activity *E. Coli*
$C_{12}H_{25}N + (CH_3)_3$	0.0228	7.50	3.6	7.50	1.5	0.033	0.033
$C_{12}H_{25}N + (CH_3)_2(C_2H_5)$	0.0213	7.50	1.8	7.50	2.0	0.035	0.035
$C_{12}H_{25}N + (CH_3)(C_2H_5)_2$	0.0199	7.50	2.2	7.50	1.5	0.038	0.038
$C_{12}H_{25}N + (C_2H_5)_3$	0.0193	7.50	1.8	7.50	2.0	0.039	0.039
$C_{12}H_{27}N + (CH_3)_3$	0.0112	2.50	1.3	2.50	15	0.022	0.022
$C_{14}H_{29}N + (CH_3)_3$	0.0058	0.750	3.7	0.750	25	0.014	0.014
$C_{16}H_{33}N + (CH_3)_3$	0.0015	0.750	0.35	0.750	2.5	0.050	0.050
$C_{18}H_{37}N + (CH_3)_3$	0.000346	0.750	0.6	0.750	0.8	0.22	0.23
$C_6H_5—N + (CH_3)_2(C_{12}H_{25})$	0.00765	1.00	10.6	1.25	12.4	0.013	0.016
$C_6H_5—CH_2N + (CH_3)_2(C_{12}H_{25})$	0.0081	1.00	9.5	1.25	11.1	0.012	0.015
$C_6H_5—CH_2CH_2N + (CH_3)_2$-$(C_{12}H_{25})$	0.0041	1.00	0.73	1.25	9.4	0.024	0.030
$C_6H_5—CH_2N + (CH_3)(C_2H_5)$-$(C_{12}H_{25})$	0.0077	1.00	10.0	1.25	12.7	0.013	0.016
$CF_3C_6H_4—CH_2N + (CH_3)_2$-$(C_{12}H_{25})$	0.0032	1.00	0.37	1.25	6.0	0.032	0.039
$C_6H_5—(CH_2)_3—N + (CH_3)_2$-$(CH_{12}H_{25})$	0.00313	1.00	1.6	...	...	0.032	...

[a] Time required for 99.99% kill.

of the experiment. When the toxic agent is applied in solution and is a substance of limited solubility, the activity at the toxic concentration can be obtained (with sufficient accuracy for judicious use) by using the ratio of the toxic concentration of the substance over its solubility.

It is the purpose of this paper to show that the Ferguson principle can be extended to the relationship of bactericidal activity of quaternary ammonium salts and the critical micelle concentration (CMC) of the salts.

DISCUSSION

J. A. Cella, *et al.* (3), have reported that bactericidal activity generally appears to increase with decreased CMC within a series of quaternary ammonium salts that were tested. The data shown in Table I are from their work.

Previous investigators (1, 2, 4) have preceded with the selection of the pure substances as the standard free state and on the basis that solutions in equilibrium with excess solid have the same thermodynamic activity. An extension of this concept should apply to the equilibrium of solutions with micelles. The thermodynamic activity and, therefore, the biological activity should be equal to a constant per cent of the CMC of the active constituent.

The activities in Table I are calculated by obtaining the ratio of the test concentration divided by CMC. The variation between activity coefficients shown in Table I is very small (with one exception), showing a two- or three-fold difference as against the 200- to 300-fold variation in the extremes of CMC. As the Ferguson principle holds here, it tends to substantiate the view that the bactericidal activity is the result of some physical activity (presumably surface effects) rather than chemical activity.

In reviewing Table I without the columns of activity indices, one would have a difficult time discerning a striking difference in the activity of any compound. However, with the inclusion of the activities it becomes apparent that compound 8 has a unique value. Therefore, this would indicate that the compound may act through a different mechanism.

SUMMARY

The thermodynamic activity values of a series of quaternary ammonium salts were calculated through the use of CMC. The correlation of these thermodynamic activities to the published values of their bactericidal activities is shown through application of the Ferguson principle.

The value of the Ferguson principle in helping to point out the possible presence of different mechanisms of activity within a series of similar compounds has been suggested.

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Pharmaceutical Investigation of Selected Alberta Bentonites III. Cation Exchange Determination

By ARTHUR J. ANDERSON† and ELMER M. PLEIN

Previously reported data established that anomolous behavior of Alberta bentonites could not be explained on the basis of official limit tests. Results of a cation exchange study of these clays are discussed.

To EVALUATE the acceptability of Alberta bentonites as pharmaceutical agents, a study of the various known deposits of the material was undertaken. Details regarding identification procedures and limit tests were reported earlier (1, 2).

Since the anomolous behavior of the clays could not be explained on the basis of the data obtained from the official limit tests, it was felt that a knowledge of the cation exchange capacities of the respective clays might provide a better basis for explaining the apparent lack of uniform quality found among the samples collected.

Clay minerals possess to a varying degree the ability to adsorb cations and to retain these in an exchangeable state (3–5). This ability is claimed to be the result of unbalanced lattices and is expressed in milliequivalents (meq.) of cation per 100 Gm. of clay. Increasing valency of the cation and decreasing hydration of the particle facilitates this exchange.

Various methods for determining the cation-exchange capacity of soils have been reported (5–12). The continued search for a satisfactory method served only to emphasize the difficulties inherent in such a determination. Even less accuracy has been found in the determination of the specific exchangeable cations (13).

EXPERIMENTAL

The procedure employed in this study was taken from the "Methods Book, Soils Survey Laboratories," University of Alberta (14), and was essentially a modified version of the ammonium acetate method of Schollenberger and Simon (12). In general, neutral ammonium acetate solution was used to displace the original adsorbed cations from the clays by a process of leaching. The leachates were retained for determination of the specific cations removed in the leaching process. The capacity of the clays to adsorb ammonium ions under controlled conditions was then determined by a Kjeldahl technique employing titration of 4% boric acid solution with standard sulfuric acid. Values obtained are presented in Table I.

In considering the ammonium acetate leachates obtained from the cation exchange procedure, it appeared that analytical procedures designed to yield quantitative values for sodium, potassium, calcium, and magnesium should be sufficient for the purposes of this study. The four cations mentioned were considered to exert the most significant effects on hydration properties of bentonites. Advantage was taken of the flame photometer analytical method for all four of the cations mentioned (Table II).

A Beckman spectrophotometer, model DU, with an acetylene and oxygen burner was employed in the determination of these exchanged cations. Sensitivity of the flame signal response was enhanced through use of a model 9200 photomultiplier unit. Interference effects were lessened by means of a flooding technique as employed by the Alberta Soils Survey Laboratories (14, 15).

RESULTS

Per cent transmittance of sodium, potassium, calcium, and magnesium was read at 589 mμ, 769 mμ, 422.5 mμ, and 285.2 mμ, respectively. The values for each ion were referred to a standard curve for that ion and translated into meq./L. This latter value was then converted into meq. per 100 Gm. of oven-dry clay through

$$\text{meq./100 Gm. oven-dry clay} = \frac{F(\text{meq./L.-blank})\ 25}{W}$$

where F = dilution factor for the leachate necessary to bring transmittance values within the range covered by the standard curve, and W = weight of oven-dry sample.

Ideally, the sum of the exchanged cations should equal the cation exchange capacity for each clay studied. However, due to a number of factors, such as errors inherent in the procedures employed plus the presence of soluble salts extracted during the leaching process, such an agreement is rarely obtained. In Table III such differences are attributed to the presence of soluble salts only.

On this basis, the clays from the Rosalind beds (Samples 1A, 3, 21, and 28) showed very little soluble salt content, with exchangeable potassium also very low. Sample 32, while also a product of

TABLE I.—CATION-EXCHANGE CAPACITY OF SELECTED ALBERTA BENTONITES

Bentonite Sample No.	Cation Exchange[a] (av.)	Range
29 (Control)[b]	82.53	0.99
1A	64.27	1.02
3	59.17	0.22
4	54.76	0.55
6	66.10	0.16
9	61.15	0.45
21	75.06	0.39
25	58.87	0.27
28	66.65	1.15
30	66.47	1.22
31	66.08	0.86
32	51.08	1.24

[a] Reported in meq. NaOH per 100 Gm. oven-dry clay.
[b] Volclay B. C. grade, received from the American Colloid Co., Merchandise Mart Plaza, Chicago 54, Ill.

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Based on a thesis presented by Arthur J. Anderson to the Graduate School, University of Washington, in partial fulfillment of Doctor of Philosophy degree requirements.
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TABLE II.—DETERMINATION OF EXCHANGEABLE IONS IN LEACHATES BY FLAME SPECTROPHOTOMETER[a]

Bentonite Sample	Na^+ 589 mμ	Range	K^+ 769 mμ	Range	Ca^{++} 422.5 mμ	Range	Mg^{++} 285.2 mμ	Range
29 (Control)[b]	50.90	2.39	2.12	0.35	56.57	2.62	13.57	0.31
1A	33.98	3.04	0.50	0.02	26.32	2.64	6.65	0.28
3	28.09	0.57	0.52	0.01	27.83	1.00	8.64	2.17
4	20.19	3.53	0.76	0.04	26.38	1.43	2.47	0.39
6	61.80	0.77	3.26	0.29	13.80	1.42	4.06	1.12
9	57.88	1.76	2.59	0.16	56.31	1.20	5.48	1.38
21	36.94	2.35	0.40	0.06	33.89	0.23	7.70	0.94
25	36.59	0.90	1.00	0.02	44.59	1.16	3.60	1.34
28	36.51	0.81	0.58	0.03	23.32	1.18	6.00	0.27
30	27.55	2.87	0.60	0.06	51.97	0.97	10.17	0.82
31	24.33	1.12	0.63	0.04	71.40	1.49	13.50	1.60
32	31.79	0.77	0.83	0.14	59.71	4.54	5.35	0.87

[a] All values are presented as meq. per 100 Gm. oven-dry clay. [b] Volclay B. C. grade, received from the American Colloid Co., Merchandise Mart Plaza, Chicago 54, Ill.

TABLE III.—COMPILATION OF CATION-EXCHANGE CAPACITY AND EXCHANGEABLE ION DATA FOR SELECTED ALBERTA BENTONITES[a]

Bentonite Sample No.	Exchanged Ions in Leachate Na^+	Ca^{++}	K^+	Mg^{++}	Exchanged Ions, Total	Cation-Exchange Capacity[b]	Soluble Salts in Clay
29[c]	50.90	56.57	2.12	13.57	123.16	82.53	40.63
1A	33.98	26.32	0.50	6.65	67.45	64.27	3.18
3	28.09	27.83	0.52	8.64	65.08	59.17	5.91
4	40.19	26.38	0.76	2.47	69.80	54.73	15.07
6	61.80	13.80	3.26	4.06	82.92	66.10	16.82
9	57.88	56.31	2.59	5.48	122.26	61.15	61.11
21	36.94	33.89	0.40	7.70	78.93	75.06	3.87
25	36.59	44.59	1.00	3.60	85.78	58.87	26.91
28	36.51	23.32	0.58	6.00	66.41	66.65	0.24[d]
30	27.55	51.97	0.60	10.17	90.29	66.47	23.82
31	24.33	71.40	0.63	13.50	109.86	66.08	43.78
32	31.79	59.71	0.83	5.35	97.68	51.08	46.60

[a] All values are presented as meq. per 100 Gm. of oven-dry clay. [b] From Table I. [c] Control: Volclay B. C. grade, received from the American Colloid Co., Merchandise Mart Plaza, Chicago 54, Ill. [d] Within the limits of error for these determinations this value may be considered as zero.

the Rosalind beds (1), had been collected from the stack at the plant in an effort to procure an air-floated commercial product free of "grit." As shown in the table, significant changes in cation values were found when compared with the "regular" Rosalind clays. A notable increase in calcium ion was found in the leachate, while a reduced cation exchange capacity was observed. On the other hand, Sample 9 with a lower swelling power and much lower gelling power than Sample 32 (2) was shown to possess a higher cation exchange capacity than the latter clay (Table III). Similar observations could be made for other samples presented, indicating that the information shown, while of interest to the clay investigation generally, was not in itself sufficient to account for the observed behavior of the clay samples studied.

This conclusion is in agreement with the findings of Foster (16) who reported that the degree of swelling of sodium montmorillonites was not correlated with cation-exchange capacity or with charge on the tetrahedral layers.

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Gas Chromatographic Data for Some Antihistamines

By ALEXANDER MACDONALD, JR., and RONALD T. PFLAUM

METHYL SILICONE POLYMER SE-30 (General Electric Co.) has been used effectively as the stationary phase in gas-liquid chromatography for the separation of alkaloids (3), sympathomimetic amines (2), barbiturates (1), and steroids (4). The separation applied to small samples uses a low liquid-to-solid packing ratio of the silicone polymer in a column operated at relatively low temperatures.

The gas-liquid chromatographic separation characteristics of 16 antihistamines have been determined using a 6-ft. column packed with 100–120-mesh Gas Chrom-P. A 1% loading factor of SE-30 was employed for the column operated at 173°.

EXPERIMENTAL

A Barber-Colman model 10 gas chromatograph equipped with an argon β-ray ionization detector and a 6-ft. packed glass column was used in this work. The column packing was prepared by the addition of a 1% benzene solution of SE-30 to a known amount of 100–120-mesh Gas Chrom-P contained in a round bottom flask. Benzene was removed under vacuum with a rotary evaporator The column was filled by the slow addition of dry packing with continuous vibration (5). The column was conditioned at a temperature of 200° for 24 hours. An argon flow of 20 ml. per minute was maintained throughout the conditioning period.

Samples of the antihistamines were prepared as 10% solutions of the free bases in chloroform. Chloroform solutions of 0.4 μl. were injected into the chromatograph with a Hamilton microliter syringe.

RESULTS

The retention times for the antihistamines studied in this investigation are presented in Table I. The data indicate that the column and operational parameters are suitable for the identification of most of the compounds listed.

The response curves obtained from the chromatograph showed sharp peaks with sharp leading edges. Tailing was a prevalent feature, becoming more pronounced with increasing retention time. Decomposition of some antihistamines was indicated by the occurrence of double peaks.

Work is in progress to overcome the tailing and decomposition problems. Other gas chromatographic procedures for antihistamines are also being investigated.

TABLE I.—GAS CHROMATOGRAPHIC DATA FOR ANTIHISTAMINES[a]

Compound[b]	*Retention Time, min.*
Antazoline (Antistine—Ciba Pharmaceutical Co.)	3.1
Carbinoxamine (Clistin—McNeil Laboratories, Inc.)	14.2
Chlorcyclizine (Perazil—Burroughs Wellcome and Co., Inc.)	20.0 22.9[c]
Chlorothen (Tagathen—Lederle Laboratories)	14.7
Chlorpheniramine (Chlortrimeton—Schering Research Corp.)	9.0 9.7[c]
Cyclizine (Marezine—Burroughs Wellcome and Co., Inc.)	1.9 8.9[c]
Diphenhydramine (Benadryl—Parke, Davis and Co.)	6.3
Doxylamine (Decadryn—The William S. Merrell Co.)	8.2
Meclizine (Bonine—Charles Pfizer and Co.)	no response
Methapyrilene (Irwin, Neisler and Co.)	8.2
Pheniramine (Dorsey Laboratories)	4.0
Promethazine (Phenergan—Wyeth Institute)	7.2 7.7[c]
Pyrilamine (Dorsey Laboratories)	25.9
Thenyldiamine (Thenfadil—Sterling-Winthrop Research Institute)	8.2
Thonzylamine (Neohetramine—Warner Lambert Research Institute)	20.9
Tripelennamine (Pyribenzamine—Ciba Pharmaceutical Co.)	7.0

[a] Column, 6 ft., 1% SE-30/100–120-mesh Gas Chrom-P; Column temp., 173°C.; detector temp., 228°C.; injection point temp., 256°C.; argon flow 60 ml./min. [b] Trade name and supplier of compound included. [c] Major peak.

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A "Go, No-Go" Assay Procedure for Residual Amounts of Acetone in Film-Coated Tablets

By WALTER W. HOLL, THEODORE P. MICHAELS, and L. PAUL SINOTTE

The iodoform reaction has been adapted as a quantitative method for the determination of residual amounts of acetone in film-coated tablets. The procedure makes use of a "Go, No-Go" principle in order to provide a rapid means of determining whether the acetone is within established limits.

TABLETS THAT are enteric coated with an acetone solution of cellulose acetate phthalate, even after considerable drying, retain residual amounts of the solvent. This residual acetone, if present in high enough concentration, results in an objectionable odor in the packaged product.

It was necessary to develop a method for the rapid determination of residual acetone and to set a limit at which no odor would be detectable. At first a colorimetric method involving formation of the 2,4-dinitrophenylhydrazone was used, and a limit of 300 mcg. per tablet established. The method, however, was time consuming (it took several hours) as it required extraction of the color from the reaction mixture before measurement (1).

For in-process control and feed-back information, a procedure that could be performed in the production area by a person with little or no technical training was desired. Instead of determining the exact quantity of residual acetone, this procedure would determine compliance with the specification limit and hence be called a "Go, No-Go" method.

The volumetric procedure that best fitted these requirements was patterned after the methods of Messinger (2) and Romijn (3). The method makes use of the familiar haloform reaction

$$CH_3-\overset{\overset{O}{\|}}{C}-CH_3 + 3I_2 + 4OH^- \rightarrow CHI_3 + CH_3COO^- + 3I^- + 3H_2O$$

Since 1 mole of acetone reacts with six equivalents of iodine, the method is suitable for measuring small amounts of acetone accurately. The determination is carried out as a back titration of excess iodine. The accuracy of this procedure has been previously reported by Goltz and Glew (4).

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The authors express appreciation to the following for suggestions and assistance in developing the concepts and data presented here: M. J. Chertkoff, C. H. Crunkleton, G. A. Slothower, G. A. Tushim, and M. H. Yunker.

TABLE I.—TESTS ON STANDARD ACETONE SOLUTIONS

Solution	Acetone Equivalent, mcg./tablet	"Go, No-Go" Result
A	297	Go
B	298	Go
C	299	Go
D	300	Go
E	301	No-Go
F	302	No-Go

EXPERIMENTAL

Reagents.—Approximately 0.1 *N* sodium hydroxide solution; concentrated hydrochloric acid; starch indicator suspension U.S.P.; standardized 0.051 *N* iodine solution; standardized 0.040 *N* sodium thiosulfate solution were the reagents utilized.

The procedure and the normalities of the iodine and thiosulfate solutions are specific for a "Go, No-Go" limit of 300 mcg. of acetone per tablet, or 0.310 meq. in the sample taken (ten tablets). The exact normalities are not critical; however, the difference (0.310 meq.) must be maintained. For other "Go, No-Go" limit concentrations of acetone, the number of meq. must be calculated and this difference maintained between the normalities of the iodine and thiosulfate solutions.

Equipment.—Automatic Palo pipets[1] of 50 ml., 10 ml., and 5 ml. delivery and standard laboratory glassware were employed.

Procedure.—Place a sample of 10 tablets in a glass-stoppered 125-ml. flask. Using Palo pipets, add 50 ml. of approximately 0.1 *N* sodium hydroxide solution and 10 ml. of the standardized 0.051 *N* iodine solution. Shake this mixture occasionally for 10 minutes or until the tablets are completely disintegrated. Add 1 ml. of concentrated hydrochloric acid. Immediately add, by means of a Palo pipet, 5 ml. of the standardized 0.040 *N* sodium thiosulfate solution.

A dark brown mixture indicates the presence of unreacted iodine and that the residual acetone in the sample is well below the limit—the sample passes (Go). If the mixture is white or pale yellow (CHI_3), the sample is above or close to the limit. In this case, add 1 ml. of starch indicator suspension. If the mixture is now blue, unreacted iodine is present and the sample passes (Go). However, if the mixture remains white or pale yellow, there is no unreacted iodine present; the acetone concentration is above the limit and the sample fails (No-Go).

[1] Marketed by Palo Laboratory Supplies, Inc., 75 Ninth Ave., New York 11, N. Y.

TABLE II.—TESTS ON TABLETS

Sample	Colorimetric Result, mcg./tablet	"Go, No-Go" Result
20	80	Go
7	269	Go
12	270	Go
19	273	Go
1	274	Go
3	274	Go
5	276	Go
2	277	Go
18	279	Go
11	283	Go
10	295	Go
4	308	No-Go
6	321	No-Go
16	324	No-Go
17	327	No-Go
14	332	No-Go
15	332	No-Go
9	443	No-Go
13	443	No-Go
8	775	No-Go

RESULTS AND DISCUSSION

The results in Table I obtained by the "Go, No-Go" test on several standard acetone-water solutions show the accuracy of the procedure to be better than ±0.5%.

Table II lists a number of tests on actual tablets and shows the correlation between the quantitative 2,4-dinitrophenylhydrazine assay and the "Go, No-Go" procedure.

These results show the test to be reliable and free from bias caused by the reaction of iodine with the tablet ingredients.

The method fulfills the original requirements which were: (*a*) it can be performed quickly (less than 15 minutes); (*b*) it can be performed by nontechnical personnel; (*c*) it can be done in the immediate production area.

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Book Notices

Basic Facts of Pharmacology. By STEWART M. BROOKS. W. B. Saunder Co., Hampton Rd., P. O. Box 850, Camden, N. J. ix + 354 pp. 15 × 21 cm. Price $5.

Written by a pharmacist and instructor of pharmacology, a reasonably concise text for the use of clinical students is presented in this work. Stress is placed on vitamins, hormones, and electrolytes because of their vital value to all segments of therapeutic practice. After a few preliminary chapters of basic knowledge, a class by class discussion of clinical applications of drugs is presented.

Infrared Spectra of Inorganic and Coordination Compounds. By KAZUO NAKAMOTO. John Wiley & Sons, Inc., 400 Park Ave. South, New York 16, N. Y. xii + 328 pp. 15 × 23 cm. Price $9.50.

A discussion and explanation of the fundamental theory of molecular vibration and the method of normal coordinate analysis is presented along with a review of the literature on infrared spectra of inorganic compounds. Compounds are classified according to molecular structure and the observed fundamental frequencies are tabulated for each group.

Problems of Psychiatry and Neurology. Vol. III. Edited by I. F. SLUCHEVSKII. Pergammon Press, Inc., 122 East 55th St., New York 22, N. Y. viii + 376 pp. 15 × 23 cm. Price £ 5.

The transactions of the Leningrad Scientific Society of Neurologists and Psychiatrists are reported. Forty papers in the usual research style are included as well as four papers dealing with clinical observations. Among topics discussed are: problem of psychic disturbances in bronchial asthma; cerebral tumors developing with symptoms of vascular diseases; application of pharmacological loads for investigation of sugar curves in disorders of the central nervous system; and treatment of epilepsy.

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TABLE II.—TESTS ON TABLETS

Sample	Colorimetric Result, mcg./tablet	"Go, No-Go" Result
20	80	Go
7	269	Go
12	270	Go
19	273	Go
1	274	Go
3	274	Go
5	276	Go
2	277	Go
18	279	Go
11	283	Go
10	295	Go
4	308	No-Go
6	321	No-Go
16	324	No-Go
17	327	No-Go
14	332	No-Go
15	332	No-Go
9	443	No-Go
13	443	No-Go
8	775	No-Go

RESULTS AND DISCUSSION

The results in Table I obtained by the "Go, No-Go" test on several standard acetone-water solutions show the accuracy of the procedure to be better than ±0.5%.

Table II lists a number of tests on actual tablets and shows the correlation between the quantitative 2,4-dinitrophenylhydrazine assay and the "Go, No-Go" procedure.

These results show the test to be reliable and free from bias caused by the reaction of iodine with the tablet ingredients.

The method fulfills the original requirements which were: (*a*) it can be performed quickly (less than 15 minutes); (*b*) it can be performed by nontechnical personnel; (*c*) it can be done in the immediate production area.

This results in considerable time saving since samples do not have to be submitted to a control laboratory, and there is no waiting period for results. Also tests can be performed during drying operations and the tablets removed from the dryer as soon as they are below the limit. This frees the dryers for other work since it minimizes oven-drying time per lot.

This "Go, No-Go" concept, commonly used in other industries, appears to have many applications in pharmaceutical quality control when applied to the chemical checking for tolerance limits only.

SUMMARY

A general chemical procedure has been presented for rapidly determining whether a product is within a specified tolerance, *i.e.*, a "Go, No-Go" procedure. As an example the procedure is applied to the control of residual acetone in film-coated tablets. This principle could easily be extended to other product ingredients and a "Go, No-Go" test performed at both the maximum and the minimum tolerances.

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The Editor comments

SELECTION OF ADVISORS FOR FDA

Considerable support has come from various quarters to the recommendation embodied in the 1962 Citizens Advisory Committee report on the federal Food and Drug Administration which suggested that the agency should have the benefit of outside advisory committees. Such panels of experts would provide counsel to FDA on the highly complex scientific questions which arise from time to time in the course of administering its responsibilities as defined under pertinent sections of the Federal Food, Drug, and Cosmetic Act, its amendments and regulations.

We endorse and support this view and urge its early and wide implementation. However, we have noted with some concern that most, if not all, discussion and consideration of this recommendation have been solely in terms of completely medically-oriented advisory bodies which would consider medical questions and problems. Our concern is that—as in the case of the *Commission on Drug Safety* about which we commented in the August issue of THIS JOURNAL—the FDA may overlook the fact that many of the problems which it now faces and will be facing in the immediate future, have pharmaceutical ramifications of major significance.

Consequently, we feel that adequate recognition of this situation is also necessary on the part of FDA officials in order that knowledgeable pharmaceutical scientists might be appointed to the committees considering medical questions. Furthermore, it is our opinion that separate committees, composed *primarily* of scientists from various appropriate disciplines, should be established to study and advise specifically on those medically-related problems about which such panels would most properly be concerned. *The fact must be recognized that many of the present complex questions concerning the safety, efficacy, and quality of drugs are basically concerned with the pharmaceutical sciences, and are only indirectly or incidentally of a medical nature.*

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Review Article

Synergism

With Special Reference to Central Nervous System Depressants

By G. VICTOR ROSSI

EXAMINATION OF THE *Papyrus Ebers* translation (126) suggests that therapists have long been intrigued by the interaction of drugs in combination. Traditionally, drugs have been prescribed in mixtures in attempts to surpass the effects of the separate components. Formulation of mixtures of active agents remained almost exclusively on an empirical basis for centuries. Indeed, the rational use of drug combinations remains a challenge to the present day medical practitioner (192).

Green (93) has aptly stated that "drug action must ultimately be explicable on a molecular basis, but owing to the complexity of living processes it is rarely possible to attribute a pharmacologic action, even qualitatively, to any precise chemical or biochemical reaction." Analyses of specific parameters are further complicated by superimposition of the action of one drug upon that of another. Nevertheless, advances are being made toward elucidation of the physicochemical bases of drug action and the fundamental mechanisms of drug interaction.

A seemingly infinite number of research publications embody, in title or text, the terms *synergism* or *potentiation*. On the assumption that certain general principles and problems relating to these phenomena might be illustrated by consideration of a selected group of pharmacologic agents, this review has been restricted primarily to central nervous system depressants. To facilitate presentation, these have been arbitrarily subdivided into three categories: analgesics, anesthetics, and hypnotics.

Nomenclature.—The ambiguity of nomenclature relative to the phenomena of drug interaction is clearly evident upon perusal of the introductory chapters of modern textbooks of pharmacology. Semantic confusion has impeded progress in various disciplines. The International Committee for the Nomenclature of Blood Clotting Factors was established with the primary objective of clarifying the chaotic terminology in this field (41). Although the problems are not comparable in scope, a need to define precisely and establish a common meaning for terms applicable to the mutual modification of drugs in combination exists.

In his classical analysis of the subject, Veldstra (207) stated that the term potentiation means "to endow with power." It was further noted that the individual components of drug combinations possess an intrinsic "power" (*i.e.*, specific activity). Therefore, in synergic combinations, "power" is not conferred, but the effectiveness of the "power" originally present may be enhanced. Inasmuch as there is no evidence of potentiation, according to the proposed definition, Veldstra recommended that use of the term in this connection be discontinued. A plethora of scientific articles published subsequent to this indictment attests to the undiminished popularity of the de-

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scriptive term "potentiation." In defense of this choice it may be noted that a more liberal definition is provided by another authority: potentiate—"to make potent or more effective" (210).

It is this reviewer's impression that the term "potentiation," as employed in the majority of cases, is intended to connote "that situation wherein one agent shows no appreciable effect on the biological system but exaggerates the response of the system to another substance" (213). However, the enhancement of response effected by a combination of an active and an inactive compound has also been designated as "sensitization" (169). Other investigators (78) have noted that a potentiator may exert its effect by impeding the metabolic transformation of the active drug or by sensitizing the organism to the drug. These authors have recommended that a compound of the first type be classified as a prolonging agent; the second, as a true potentiator.

The heuristic merit of the word "potentiation" is perhaps as acceptable as any other. Because of the diversity of current interpretations, however, the term will be meaningless unless the individual author clearly delineates the concepts implied by its use.

In this review the general term "synergism" will be used in reference to all situations wherein facilitation of a pharmacologic response is obtained by the combined action of two or more compounds. Although some authors have defined synergism in a more restricted sense, the term (Gk. *synergos*) literally means "working together" (210), cooperation. For clarity of expression, the concept of synergism as translated into molecular terms by Veldstra (207) warrants repetition: "the combination effects a certain response with a smaller number of molecules than that required for the most active compound separately, or in the range of suboptimal concentrations, the effect of a certain number of molecules of this compound is enhanced in the mixture."

ANALGESICS

The ideal of a potent nontoxic and nonaddictive analgesic awaits realization. New molecules have not evidenced satisfactory dissociation of the seemingly obligatory relationship between analgesia and toxicity (159). Thus, other means of compensating for the deficiencies inherent in currently available compounds have been explored.

Narcotic Antagonists.—One approach to the optimal utilization of analgesics has been directed toward the concomitant administration of a narcotic and a narcotic antagonist. In one sense, narcotic antagonists may be considered as "selective synergists." Nalorphine and levallorphan inhibit many (but not all) of the pharmacodynamic effects of morphine and other narcotic analgesics (150). Their remarkable antidotal effect on narcotic-induced respiratory depression suggested the possible clinical utility of narcotic: antagonist mixtures in the alleviation of severe pain. Despite early favorable reports, the use of morphine: nalorphine combinations has not gained wide acceptance. In controlled studies involving postoperative patients and healthy volunteers, a combination of 2 mg. of nalorphine and 10 mg. of morphine was found to produce analgesia and side-effects indistinguishable from those achieved by 10 mg. of morphine (10, 124). Houde and Wallenstein (99) observed that the incidence of side-effects increased in direct proportion to the relative concentration of nalorphine in morphine : nalorphine mixtures administered to hospitalized cancer patients. Analgesic: levallorphan combinations have been viewed with somewhat greater favor. Cullen and Santos (50) used a premixed solution of levorphan tartrate and levallorphan tartrate in an 8:1 or 10:1 ratio for the relief of chronic severe pain. The analgesic effectiveness of the combination was considered essentially equivalent to levorphan, whereas the respiratory depression was significantly less than that produced by the narcotic alone. Foldes, *et al.* (75), reported that levallorphan effectively blocked the respiratory depressant action of alphaprodine when these compounds were injected simultaneously for the purpose of obstetrical analgesia. Comparable results were obtained with oxymorphone: levallorphan mixtures (76).

The numerous clinical studies involving combinations of narcotics and narcotic antagonists have been critically reviewed by Eckenhoff and Oech (66). These authors hold that narcotic depression of respiration may be minimized by prior or simultaneous administration of a specific antagonist in the alleviation of acute painful conditions. In their opinion, however, such mixtures are not applicable to the treatment of chronic pain. This limited therapeutic efficacy of narcotic: antagonist combinations is not conceded by Telford and Keats (201).

Synergists.—Another approach to the goal of improving the efficacy of existing analgesic drugs has involved their use in combination with a variety of essentially unrelated compounds found, largely by "trial and error," to augment

analgesic activity. Both adrenergic (89, 102) and cholinergic (111, 112, 178) drugs have been reported to enhance the effectiveness of morphine. Veldstra (207) has documented the extensive literature relating to enhancement of the analgesic activity of narcotics by such diverse agents as suramin, sparteine, antispasmodic agents, and several methonium compounds. Recent studies have demonstrated that the activity of narcotic analgesics in experimental animals may also be augmented by captodiamine (113), carbetidine (143), chlortetracycline (145), mephenesin (170), mephenoxalone (92), methylquizolone (14), pentolinium (94), phenetamine (131), quinine (157), reserpine (51), tryptamine, 5-hydroxytryptamine, amphetamine and mescaline (187), and SU-8629 (2-amino-indane HCl) (216). Data supporting the clinical efficacy of these combinations is lacking, however.

SKF 525-A.—With the exception of a hypocholesterolemic effect demonstrated in several animal species (56), SKF 525-A (β-diethylaminoethyl-diphenylpropylacetate HCl) possesses minimal direct pharmacologic activity (23). Primary interest in this compound has centered on its intriguing ability to act synergistically with drugs of diverse chemical structure and pharmacodynamic properties. Among the different types of drugs whose characteristic activities have been shown to be augmented by SKF 525-A are included: barbiturates and nonbarbiturate hypnotics (44), analgesics (45), antiepileptic drugs (195), spinal cord depressants and central nervous system stimulants (136), skeletal muscle relaxants (153), antihypertensive agents (90), and certain antibacterial and antiprotozoal drugs (202).

The key to the practicality of synergistic drug combinations is selectivity. Nonselective enhancement of the entire pharmacodynamic spectrum of a given drug provides a doubtful therapeutic advantage. Optimally, an augmentation of the therapeutically useful properties of the active component, accompanied by suppression of undesirable effects, is achieved. Exploration of the mechanisms of selective synergy remains to be exploited as a possible avenue to the objective of improved pharmacotherapy.

Cook, *et al.* (45, 46), showed that SKF 525-A enhanced the analgesic effect of various narcotics in rats without influencing either the LD_{50} (of morphine or meperidine) or the respiratory suppressant action (of morphine) in the same animals. This clearly demonstrated that different facets of the narcotic spectrum could be influenced selectively. Although the synergistic agent in this case (SKF 525-A) has proven an extremely useful tool in the investigation of pathways of drug metabolism, it remains restricted to experimental use.

It may be of interest that chronic administration of SKF 525-A is associated with hepatotoxicity in rats (204) and dogs (56). Holmes and Bentz (96) have postulated that interference with an important electron transfer coenzyme (coenzyme Q) may account for SKF 525-A-induced accumulation of lipid material in the livers of animals.

Phenothiazines (Animal Studies).—In the initial report on the subject Courvoisier and her associates (49) noted that chlorpromazine, although devoid of intrinsic analgesic activity, increased the intensity and prolonged the duration of action of morphine in mice. Subsequently, several investigators, using a variety of analgesimetric techniques, obtained comparable results in experimental animals with chlorpromazine and other phenothiazine derivatives (51, 55, 81, 152, 154, 162, 180, 205). The statement that the phenothiazines are not effective pain-relieving drugs, but that they potentiate the activity of specific analgesics, reflects the current consensus. Both of these views represent oversimplifications based largely on the original studies with promethazine and chlorpromazine.

Carter and David (38) reported that preinjection of chlorpromazine, thioperazine, and prochlorperazine effectively augmented the analgesic activity of morphine, meperidine, phenazocine, and raceoramide (SKF 5137) in rats. Of these three phenothiazine derivatives, chlorpromazine and thioperazine were found to diminish the degree of tolerance, but not necessarily the rate at which tolerance developed, when the narcotics were administered once daily during a 9-week period. Comparable enhancement by chlorpromazine of the analgesic activity of morphine in rats was observed by Mazurkiewicz and Lu (140). However, these investigators noted that chlorpromazine did not retard the development of tolerance in rats given a single daily injection of the narcotic during a period of 7 weeks.

Weiss and Rossi (212) reported that preinjection of a trifluorinated phenothiazine derivative (NDR-3680) enhanced the analgesic activity of codeine, dihydrocodeine, and morphine (d'-Amour-Smith analgesimetric method), and inhibited the constipating and respiratory depressant effect of codeine and morphine, but not dihydrocodeine, in rats. These investigators also found that phenothiazine pretreatment increased the extent and duration of protection provided by codeine, dihydrocodeine, and morphine against phenylquinone-induced writhing, reduced the

acute toxicity of codeine and dihydrocodeine, but increased the toxicity of morphine in mice.

Although there have been many differences in magnitude and a few differences in direction of the response, investigations performed with experimental animals have provided more consistent evidence of a synergistic relationship between the phenothiazines and the analgesic activity of narcotics than those studies performed in man.

Phenothiazines (Human Studies).—Numerous publications have appeared which describe the effects of phenothiazines, alone and in conjunction with barbiturates or analgesics, in surgical and obstetrical situations (82, 114, 137, 188, 191, 211), and in chronic pain of diverse origin (132, 174, 208). The lack of uniformity of rating systems, the paucity of control series, and the failure to provide statistical validation of the significance of the difference between treatments, mitigates against acceptance of facile claims of phenothiazine "potentiation" characteristic of many reports in this category. This reviewer is cognizant of the inordinate difficulties encountered in the evaluation of drugs intended for the amelioration of subjective phenomena in humans. However, these difficulties cannot always be accepted as an adequate excuse for poor experimental design. Controlled studies often reveal astonishing gaps between clinical impressions, theoretical assumptions, and established facts. Beecher (9), Wolf (217), and Modell and Houde (147) hold that there are no compelling reasons why quantitative methods cannot be utilized in the evaluation of subjective responses in humans.

Boulton (16) reported that the administration of chlorpromazine (25 mg. orally), prior to and following anesthesia, reduced the postoperative requirements of meperidine by 23% in females and by 6 to 7% in males who had undergone thoracic surgery. Dyrberg and Johansen (64) found no difference in the postoperative requirements for analgesic drugs among patients premedicated with 10 mg. of morphine or 50 mg. of chlorpromazine. Addition of 5 mg. of perphenazine to the standard premedication was found by Phillips, *et al.* (161), to reduce the incidence of requirements of analgesic drugs in the postoperative period by approximately 50%. Comparable results were obtained by Lear, *et al.* (128), with triflupromazine. The variables involved in such clinical situations make it extremely difficult to dissociate the effects of phenothiazines *per se* on anxiety reactions, and the possible enhancement of the analgesic activity of narcotics as distinct from prolongation of the action of general anesthetics.

Sadove, *et al.* (174), reported that narcotics and sedatives, which had previously proven ineffective in 22 of 28 patients with malignant lesions, provided satisfactory relief of pain when given in conjunction with chlorpromazine. Wallis (208) concluded that chlorpromazine effectively augmented the action of narcotics in the majority of patients suffering chronic pain of diverse origin. Light, *et al.* (132), noted that promethazine enhanced and prolonged the effectiveness of meperidine and morphine in two patients suffering the pain of inoperable malignancy. In a controlled crossover study, Houde and Wallenstein (98) found that single doses of 25 mg. of chlorpromazine alone exhibited no significant analgesic effect in 34 hospitalized patients with chronic severe pain. Furthermore, the relief obtained with a combination of morphine sulfate (10 mg.) and chlorpromazine (25 mg.) was essentially the same as that provided by the narcotic alone in these patients.

Respiratory and Circulatory Effects.—In a discussion of combination therapy, Sollmann (189) stated that "synergism is utilized to secure the summation of the desirable effects of several drugs, while the side actions are not increased in proportion, or may even be neutralized." Several experimental animal studies noted in this review have demonstrated that the simultaneous use of two drugs may result in a selective reinforcement of certain pharmacodynamic properties with a resultant increase in the therapeutic index. However, few clinical data substantiate that such a fortuitous situation applies to the combined use of phenothiazines and narcotics in humans.

Divergent clinical reports indicate that phenothiazines suppress (127, 166), intensify (122), or do not significantly alter (68, 80, 108) the effects of narcotics on respiration. Obviously, the influence of phenothiazine derivatives on narcotic-induced respiratory depression in humans remains equivocal. Data provided in support of phenothiazine antagonism of the respiratory effects of narcotics are not particularly convincing, however.

The alterations of circulatory homeostasis by phenothiazine derivatives and by narcotic analgesics, separately, is well documented. Reduction of systemic arterial pressure by the phenothiazines is apparently due to a combination of factors, including depression of the vasomotor regulatory centers (198), adrenergic blockade (88), and direct relaxation of arterial smooth muscle (73). The hypotensive activity of narcotic analgesics may also be attributed to multiple mechanisms, which include inhibition of the vasomotor centers (70), and peripheral vasodilation

due to a direct effect on the blood vessels (179), or mediated *via* histamine liberation (72). Variations are considerable among the different members of each series; however, the phenothiazines generally manifest greater vasodepressor potency than the narcotics. The theoretical assumption was made by Burgi (207) that "in combining drugs with the same end effect, the resulting activity is additive when the sites of action of the components are identical and superadditive if they are different." Although this generalization is not universally applicable, the complex of mechanisms associated with the hypotensive effect of the phenothiazines and the narcotics would appear to provide an ample basis for synergistic activity. Data supporting this contention are lacking; however, Eckenhoff and Oech (66) consider that the combination of a narcotic with a phenothiazine derivative theoretically imposes a greater threat to the circulation than it does to the respiration.

Qualitative Differences.—The "drug explosion" (146) has added impetus to a pre-existing tendency to group structurally and/or pharmacodynamically related chemicals into a common category. The phenothiazines represent a case in point. Exaggerated emphasis has been placed on the phenothiazine nucleus as a determinant of biodynamic activity; the majority of published reports foster the impression that the various substitutions largely influence the properties of the molecule in a quantitative rather than a qualitative sense. Increasing sophistication has resulted in dissatisfaction with such generalizations.

The property of synergy has become identified indiscriminately with the phenothiazines as a group, although this association does not hold necessarily for all members of the series. Possible explanations for selectivity in regard to the enhancement of analgesic activity may be discerned in those studies which have demonstrated that the phenothiazines cannot be considered as a homogenous group with respect to their effect on pain.

Analgesic versus Antianalgesic Activity.—Hougs and Skouby (100), using the Hardy, Wolff, and Goodell radiant heat analgesimetric method, noted that chlorinated phenothiazine derivatives exerted a mild analgesic action which was not demonstrable with the nonchlorinated compounds. Boreus and Sandberg (15), who also employed a thermal technique for inducing pain in healthy human volunteers, concluded that although chlorpromazine and acetylpromazine reduced somewhat the sensitivity to noxious stimulation, neither significantly increased the analgesic action of methadone. Mepazine, which evidenced no analgesic action when administered alone, antagonized the pain-relieving effect of methadone.

Methotrimeprazine was found to be equivalent (on a milligram basis) to morphine in providing relief of pain in postoperative and postpartum patients (125). Promazine, chlorpromazine, and trimeprazine evidenced modest effectiveness against experimentally induced pain in humans, whereas other phenothiazine derivatives manifested "slight" (prochlorperazine, perphenazine, trifluoperazine, triflupromazine) or "marked" (promethazine, mepazine) antianalgesic activity (149). Methdilazine, despite a marked sedative propensity, significantly increased the amount of meperidine required for the control of postoperative pain (200). Thus, contrasted to other investigators (133) who reported an augmentation of the activity of morphine and meperidine by methdilazine (radiant heat analgesimetric method in rats), Taylor, *et al.* (200), concluded that this phenothiazine derivative manifested a definite analgesic antagonism. It is possible that these apparently divergent conclusions are both valid, but that one is encountering the classical question of the relationship between the perceptual and psychic components of the pain phenomenon in man and the reflex reaction patterns to noxious stimuli in laboratory animals (109).

This dichotomous effect on patient sensitivity to pain may be reflected in the degree to which the action of the central nervous system depressants can be influenced by premedication with different phenothiazine derivatives. Dundee and Moore (62) found that, with the exception of those derivatives which evidenced a marked antianalgesic action, the phenothiazines under consideration significantly reduced the total dosage of barbiturate (methohexitone) required during the performance of a standard surgical procedure. The increased need for supplementary doses of barbiturate following premedication with promethazine and mepazine suggested the possibility of an antagonism between the antianalgesic phenothiazines and the anesthetic state.

Other evidence suggests that categorization of the various phenothiazine derivatives on the basis of an "analgesic" or "antianalgesic" effect may be artefactual. Clinical studies have indicated that barbiturates may have a diphasic effect on sensitivity to pain in man (4). Relatively high concentrations induce an anesthetic state *per se* or augment the analgesic or anesthetic activity of other agents. In contrast, a reduced threshold to pain may be experienced in the

presence of low levels of barbiturate in the central nervous system. A comparable diphasic action in regard to analgesia may be characteristic of other central depressants, *viz.*, phenothiazines (63). It is within the realm of possibility that, depending on dosage and/or time parameters, a particular phenothiazine might manifest either a synergistic or an antianalgesic effect.

ANESTHETICS

Using mice as the test subjects, Carson and Domino (37) found that relatively large doses of chlorpromazine (15 mg./Kg., intraperitoneally) significantly reduced the mean time required for loss of the righting reflex during exposure to ethyl ether, chloroform, or halothane. However, an equal dose of promethazine did not alter the time required for onset of anesthesia with these volatile agents. Brunaud, *et al.* (28), showed that propiomazine was considerably more effective than promethazine in its ability to prolong ether anesthesia in mice. Perhaps because of the technical difficulties associated with the quantitative administration of volatile anesthetics to small animals, most of the experimental animal studies in this field relate to effects obtained with fixed anesthetics (*i.e.*, barbiturates). Although the division is arbitrary, these reports will be considered in the section on *Hypnotics*.

Awareness of the potential application of phenothiazines in surgical anesthesia stemmed largely from the publications of Laborit and his coworkers (115, 116, 118–120), particularly in regard to promethazine and chlorpromazine. The role of this group in the development of the concept of "potentiated anesthesia" has been detailed by Laborit (117).

The versatility of phenothiazines in surgery is characterized by the reports of Sadove (173) and Dobkin (58), who describe the use of promethazine as a sedative preoperatively, as a supplement to anesthetics in producing the lighter planes of anesthesia and the hypothermic state, and as a means of combating emesis and hiccups. Taylor, *et al.* (199), observed that premedication with chlorpromazine alone permitted surgical anesthesia to be obtained with significantly lower concentrations of ethyl ether than with atropine alone. Carroll and Moir (35), and Adelman, *et al.* (1), reported that premedication with promethazine facilitated the induction and maintenance of general anesthesia and appeared to reduce the requirements of anesthetic agents in surgical and obstetrical cases. Stone (193) found that trifluprom azine facilitated the action of general anesthetics in doses which did not usually depress circulation. Thiopental anesthesia in man has been found to be augmented by a variety of central nervous system depressants, including antiemetics, antihistamines, and phenothiazine and nonphenothiazine ataraxics (60).

HYPNOTICS

Drowsiness associated with the clinical use of many antihistaminic drugs has been observed repeatedly. In experimental animals, however, it is ordinarily not possible to demonstrate sedation by the antihistamines when administered alone. In 1948, Winter (214), proceeding on the assumption that a simultaneous central excitatory action might mask a covert sedative effect, found that where antihistamines *per se* did not grossly manifest central depressant activity, they significantly prolonged the hypnotic action of hexobarbital in mice and guinea pigs. Although the basic technique had been previously employed by other investigators (34), Winter's report (214) catalyzed a surge of interest, which continues unabated in the potentialities of this tool.

Determination of the ability of a compound to prolong the duration of the loss of the righting reflex induced by a hypnotic dose of a barbiturate (generally hexobarbital) in laboratory animals (generally mice) remains a procedure widely used on the assumption that it provides a means of detecting subtle components of central nervous system depressant activity. The most frequently employed modification of this procedure is based on determination of the ability of a compound to convert a subhypnotic dose of a barbiturate to one which will induce a loss of the righting reflex (168, 206, 218).

There are few substances whose activity, based on either one or both of these criteria, has escaped examination.[1] Early studies relating to prolongation of barbiturate hypnosis, with particular reference to the antihistamines, disulfiram, carbohydrate metabolites, SKF 525-A, and alpha-tocopherol phosphate, have been previously reviewed (207). Within the past several years a representative grouping of compounds of diverse structure which have been reported to enhance the action of barbiturates and other hypnotics, includes: chlorpromazine, promazine, and their metabolites or "model" metabolites, monomethylchlorpromazine, chlorpromazine-N-oxide, 2-hydroxypromazine and 4-hydroxypromazine (163), other phenothiazine derivatives

[1] Only selected reports concerning hypnotic drug synergism are cited in this review. A separate bibliography relative to the subject and containing in excess of 100 references is available on request.

(28, 133, 196), reserpine and other rauwolfia alkaloids (27), urethan (83), chloral hydrate (71), diphenylhydantoin and other anticonvulsant drugs (74), captodiamine (113), amphenidone, hydroxyzine and meprobamate (167), phenaglycodol and metaglycodol (209), emylcamate (141), metaxolone, carisoprodol, and benactyzine (36, 97), various thiaxanthene derivatives, including chlorprothixene (148), meperidine (139), morphine and other analgesics (54), and many other compounds evidencing overt central nervous system depressant activity. To this list of drugs, which would be expected to add to the depressant effect of barbiturates or other hypnotics, may be appended a heterogeneous group of compounds, all of which have been reported to augment sleeping time: N-acetyl-*p*-aminophenol (19), aminopyrine and phenylbutazone (67), octamethylpyrophosphoramide and other anticholinesterase agents (72), quinine (157), atropine and scopolamine (142), colchicine (8), chlortetracycline (145) and chloramphenicol (57), thyroxin (43), epinephrine, norepinephrine, ephedrine, and other adrenergic amines (144), nikethamide (168), and the antidepressant compounds, imipramine and amitriptyline (95), iproniazid and other amine oxidase inhibitors (69, 123).

Methods.—Assays based on (*A*) prolongation of sleeping time, and (*B*) conversion of a subthreshold dose of a hypnotic to one which will result in loss of the righting reflex, have previously been cited as those most commonly employed (with kaleidoscopic nuances) in studies of hypnotic drug synergism. Another perspective may be gained by a third procedure based on (*C*) reinduction of hypnosis after spontaneous arousal from anesthesia. The latter technique is used less frequently; therefore, relatively little data are available on which to base comparisons among the three methods.

The heterogeneity of molecules which have been found to extend the duration of barbiturate-induced sleep underscores the grossly nonspecific nature of this assay. Although it is of limited utility in attempts to discern qualitative differences, it is of value when used conjointly in the establishment of pharmacodynamic profiles, and it may be considered a versatile investigative tool. One ponders, nevertheless—to what extent has the magnificent simplicity of this "data machine" (major components: mouse, stopwatch) contributed to its ascendancy?

Interest in the ability of a compound to prolong the sleeping time induced by a hypnotic dose of a barbiturate (method *A*) resided initially on the supposition that the assay provided a rapid means of detecting elusive central depressant activity, *e.g.*, in the evaluation of potential sedative or ataractic drugs. In a large number of cases the mechanism involves primarily (*a*) a summation of the central depressant effects of the drugs in question. It is apparent, however, that other factors, acting independently or conjointly, may also be responsible for the effect observed; these include: (*b*) increased rate of penetration into the central nervous system, (*c*) decreased binding to plasma or tissue components, *i.e.*, loss at "silent receptors," (*d*) reduced rate of biotransformation or (*e*) excretion, (*f*) contribution of the postictal depressant component of central stimulants, (*g*) "sensitization" to central depressant drugs as a consequence of alterations in acid-base balance or brain electrolyte patterns, etc. In the absence of other types of supporting data, it is obviously difficult to discern which of these factors (or conceivably other mechanisms not cited) are operative.

Some investigators (11) have used induction time of barbiturate hypnosis (*i.e.*, interval between injection and loss of righting reflex) as the criterion for alteration in the permeability of the "blood brain barrier." While this parameter may serve as a tentative indication of permeability changes, Child, *et al.* (39, 40), have shown that a decrease in induction time does not necessarily imply an increase in the rate of penetration of barbiturates into the brain.

Methods *B* and *C* are fundamentally related in that they both involve the ability of a compound to induce a loss of the righting reflex in the presence of a subhypnotic concentration of a barbiturate (or other hypnotic) in the blood. Method *C* constitutes the most rigorous challenge inasmuch as the second agent is not administered until after the brain concentration of barbiturate has receded below the anesthetic threshold, as evidenced by spontaneous arousal. Enhancement of barbiturate activity in all three situations depends not only on the inherent properties of the synergist but also on the dose administered, route of administration, and latency of action. These latter considerations are more critical in method *B* than in method *A*, and are most critical in method *C*, particularly in the presence of a hypnotic having a short biological half-life (*e.g.*, hexobarbital). Dismissal of these factors from consideration could conceivably lead to the adoption of erroneous conclusions regarding probable mechanisms of synergy.

The isobolometric studies of Loewe (134) provided discrete evidence of the dissociability of intensity of effect and duration of effect regarding barbiturate synergism. He found that the

HD_{50} (median hypnotic dose) of pentobarbital was essentially unchanged by any dose of strychnine, whereas the HD_{50} of butallylonal was diminished by approximately 50% by a wide range of strychnine doses in mice. Irrespective of the alteration of hypnotic threshold, strychnine prolonged the duration of sleeping time with both barbiturates.

Shagass (184) developed the concept of sedation threshold, a determination of the amount of barbiturate required to produce certain quantitative changes in the electroencephalogram, as a possible index of emotional tension in humans. In a related study of thiopental thresholds in rabbits, using "head drop" as the behavioral end point, Shagass, *et al.* (185), noted that chlorpromazine significantly diminished the amount of thiopental required to elicit the characteristic response. This procedure should be explored as a possibly sensitive and quantitative approach to the evaluation of barbiturate-synergist interactions.

Lessin (129) proposed a battery of three pharmacologic assays in mice to estimate inhibition of drug oxidation based on the assumption that a "nonspecific" liver oxidase system serves as the common denominator in: (*a*) prolongation of pentobarbital hypnosis, (*b*) intensification of chlorpromazine-induced hypothermia, and (*c*) reduction in the acute toxicity of octamethylpyrophosphoramide. Qualitatively similar results were obtained with isoniazid, iproniazid, and SKF 525-A in these three assays. It is an interesting approach; the validity of the hypothesis should be supported by examination of a larger series of compounds.

Variations in Response.—The many quantitative and occasional qualitative variations in response as they relate to species, sex, and age differences in the metabolism of drugs have been recently reviewed by Bousquet (17) and Brodie (22). Particularly relevant to this subject are the studies of Quinn, *et al.* (164), who demonstrated a striking relationship between hexobarbital sleeping time and the biological half-life of this barbiturate in several species. These investigators noted, however, that following administration of hexobarbital, mice, rats, and rabbits recovered the righting reflex at plasma levels of approximately 60 mcg. of barbiturate per ml., whereas hypnosis in dogs and man persisted until the level had declined to about 20 mcg. per ml. These data suggested that, in addition to differences in rates of drug metabolism, variations in the sensitivity of the central nervous system play a major role in species differences in response to depressant drugs. Other factors germane to the interpretation of barbiturate synergism studies are the possible effects of hypothermia and hydration.

Hypothermia.—Studies conducted with a series of antihistamines demonstrated the absence of a relationship between histamine antagonism and synergic activity with barbiturates (2, 3). This conclusion was corroborated by the finding that histamine also prolonged barbiturate sleeping time. Although distinct quantitative differences were evident, Packman, *et al.* (158), found that histamine and each of 15 different antihistaminic compounds evaluated reduced the body temperature of mice. Subsequently, other investigators (71, 130, 218) reported that many drugs which prolong the action of hypnotics also lower body temperature. Conversely, β-tetrahydronaphthylamine, a potent pyretic agent, inhibited the prolonging action of 5-hydroxytryptamine on hexobarbital sleeping time in mice (160). There is little evidence of a strict causal relationship; nevertheless, the influence of a drug on body temperature is frequently a determinant of its effect on barbiturate hypnosis. Examination of the literature relative to barbiturate synergism suggests that not all investigators are aware of the importance of ambient temperature and the possible hypo- or hyperthermic effects of the compounds being studied on the response to hypnotic drugs.

Hydration and Dehydration.—Bhide (13) reported that hydration (intraperitoneal injection of water or 5% glucose solution) significantly increased hexobarbital sleeping time in mice. This finding was confirmed by Ramwell and Lester (165), who also observed that dehydration (resulting from deprivation of water or injection of various diuretics) markedly reduced the duration of hexobarbital hypnosis. The effect of water loading on the brain electrolyte pattern and electroshock seizure threshold has been detailed by Swinyard (195), for the experimental evaluation of anticonvulsant drugs. However, relatively little attention has been accorded the possible influence of alterations in the extracellular sodium concentration on the duration of barbiturate hypnosis. It is reasonable to speculate that changes in the electrolyte balance may constitute a critical factor in the observed effect of corticotropin and corticosteroids on sleeping time (215). The susceptibility of mice to dehydration should also be considered in the design of sleeping time experiments.

Dose and Time Factors.—Most investigators are cognizant of the limited value of data which relate the biological effect of a compound to a single dose evaluated at an arbitrarily selected

time interval after administration. Construction of dose-time-effect relationships requires serial determinations of response repeated in temporal sequence at various levels in the range from the minimum to the maximum effective concentration. However, practical considerations frequently restrict the completeness with which three-dimensional analyses are performed with individual compounds. Considering the additional complexities inherent in the pharmacologic evaluation of drug mixtures, it is not surprising that most studies in this area lack adequate perspective.

The observation of Shore, *et al.* (186), that relatively large doses (10 mg./Kg.) of lysergic acid diethylamide (LSD) antagonized a central action of 5-hydroxytryptamine (5-HT) in the intact animal, provided a basis for speculation as to the role of this amine in normal and disturbed mental processes and the mechanisms of action of psychosomimetic and psychosoplegic drugs. It is not within the scope of this review to consider the controversies in these areas; however, it is relevant to note certain apparently dose-related inconsistencies which have necessitated a re-evaluation of earlier concepts. LSD and 2-brom-*d*-lysergic acid diethylamide (BOL), in doses (2 to 5 mcg./Kg.) which did not alter the duration of hexobarbital hypnosis in mice, were found to enhance further the prolonging effect of 5-HT (but not reserpine) on hexobarbital sleeping time (175). Larger doses (2.5 to 80 mg./Kg.) of LSD, which have been claimed both to prolong (33, 219) and have no effect (27) on barbiturate hypnosis, and larger doses of BOL, having no effect (33) in this regard, blocked the prolongation of sleeping time induced by either 5-HT or reserpine (176) but not iproniazid (27).

In certain cases, either a synergistic or an antagonistic relationship may be effected, depending upon the time interval separating administration of two drugs. Prolongation of barbiturate-induced hypnosis by chlorpromazine, administered simultaneously or several hours prior to the hypnotic, is well documented. In contrast, Kato (105) showed that sleeping time was significantly reduced when pentobarbital or hexobarbital was administered 48 hours after chlorpromazine (15 mg./Kg., intraperitoneally) in rats. A comparable diphasic effect on the duration of hexobarbital action has been reported to occur with urethan (83), SKF 525-A, N-ethyl-3-piperidylbenzilate HCl, nikethamide, and iproniazid (183). Other studies have demonstrated a marked reduction in the duration of action of pentobarbital, hexobarbital, meprobamate, and zoxazolamine in rats injected 24 hours or more in advance with a variety of drugs and chemical carcinogens (17, 20, 42). The decreased sensitivity is explained by an accelerated *in vivo* biotransformation, subsequent to the increased activity of hepatic microsomal drug-metabolizing enzymes induced by a host of foreign compounds (85).

Recent evidence suggests that SKF 525-A and Lilly 18947 (2,4-dichloro-6-phenylphenoxyethyl diethylamine HBr) may have an "immediate" inhibitory and a "late" inducing effect on the same enzyme systems (106, 107). The interesting hypothesis has been formulated that the initial inhibitory action may be the factor responsible for stimulating the compensatory increase in *de novo* biosynthesis of microsomal drug-metabolizing enzymes (107). Further studies are needed on the relationships between the inhibitory action and enzyme-inducing action of other compounds; in some cases, synergism and antagonism may be different sides of the same coin.

MECHANISMS

The number of different types of drug combinations for which a synergistic relationship has been claimed greatly exceeds the plausible explanations of the mechanisms involved. At present no unifying concept appears adequate to account for all the diverse forms of molecular interaction encompassed by the term "synergism." Veldstra (207) suggested that a large proportion of the cases may be explained on the basis of a competition between the active compound and the synergist at various "sites of loss" for the former. These sites of loss have been identified as loci of nonspecific adsorption (*i.e.*, storage at "silent receptors"), as enzyme surfaces functioning in the metabolism or detoxication of the active compound, or as excretion mechanisms. Although this pregnant concept provides a valuable frame of reference, it is obvious that detailed analyses are required to elucidate specific mechanisms of synergic activity.

Structure-Activity Relationships (Phenothiazines).—Adequate data are not available to establish clear relationships between chemical structure and synergic activity of the various phenothiazines, although some efforts in this direction have been reported. Dobkin (59) evaluated several phenothiazines on the basis of their ability to prolong thiopental anesthesia in the dog. Quantitative relationships could not be derived inasmuch as different doses of the various derivatives were used. Nevertheless, in the doses employed, promazine and propiomazine were most effective in prolonging the anesthetic

effect of thiopental; levomepromazine and methdilazine were somewhat less active, whereas mepazine, prochlorperazine, and trifluoperazine did not influence the duration of thiopental anesthesia.

A comparative study of seven phenothiazine derivatives revealed the lack of a definite relationship based on their ability to prolong barbiturate hypnosis in mice, and impair locomotor activity in rats, and climbing dexterity in mice (177). The outstanding exception in this case, perphenazine, exhibited the greatest activity in all three assays. Jindal, *et al.* (103), compared the effects of uniform doses (on a milligram basis) of a series of phenothiazine derivatives on the duration of pentobarbital-induced hypnosis in mice. The interesting aspect of this study is that compounds which prolonged sleeping time also evidenced antidiuretic effects in saline-loaded rats, whereas those compounds which either reduced or did not alter the duration of pentobarbital hypnosis had a diuretic effect in such rats. The authors suggested that a parallelism may exist between these two phenomena. Domino (61) stated that it is unlikely that antidiuretic hormone release is the only factor involved in the enhancement of pentobarbital anesthesia by phenothiazines, but it may be contributory.

Biochemical Mechanisms (Phenothiazines).—Bain and Mayer (7), and Laborit, *et al.* (121), have prepared comprehensive analyses of researches relating to the effects of chlorpromazine and certain other phenothiazines on various enzyme systems. There is a progressively increasing literature documenting the action of phenothiazines as relatively nonspecific inhibitors of many biochemical reactions *in vitro*. However, inferences are frequently drawn from *in vitro* experiments regarding the effects of drugs on enzymatic activity, with little consideration given to the influence of normal cellular architecture on biochemical processes *in vivo*. At present it is difficult to relate the pharmacodynamic actions of the phenothiazines to their currently established biochemical effects. Despite the relatively low potency evidenced in many of the *in vitro* systems, it is nevertheless tempting to speculate that inhibition of drug metabolism constitutes at least one aspect of the mechanism of phenothiazine synergy.

Decsi (53) compared a variety of central nervous system depressants, including eight phenothiazine derivatives, on the basis of their ability to inhibit a conditioned avoidance response in the rat, augment the hypnotic action of hexobarbital in the mouse, and inhibit oxidative phosphorylation and adenosine triphosphatase activity in rat brain homogenates. The investigator concluded that there exists a quantitative relationship among the biochemical and pharmacologic effects described, thus enabling prediction of the *in vivo* potency of a drug solely on the basis of its *in vitro* activity. Inspection of the data failed to reveal adequate justification for this confidence.

Chlorpromazine was found, in healthy humans (194) and in rabbits (203), to elevate the blood alcohol concentration significantly above the levels detected in control series after adminstration of a standardized dose of ethanol. Although the study of Zirkle, *et al.* (220), did not substantiate the effect of chlorpromazine on the blood alcohol level, it demonstrated that chlorpromazine markedly increased the impairment of neuromuscular coordination following a standard dose of ethanol in man. This apparent discrepancy may be related to the indirect estimation of blood alcohol volume by means of a "Breathalyzer" in the latter study. Tipton, *et al.* (203), concluded that the elevation of the blood alcohol level by chlorpromazine was not due to an increase in the rate of absorption, but that it may be attributable to an inhibition of ethanol metabolism. In support of this premise, Khouw, *et al.* (110), have demonstrated that relatively low concentrations of chlorpromazine inhibit the activity of rabbit liver alcohol dehydrogenase.

Kaplan, *et al.* (104), demonstrated that large doses of nicotinamide markedly increased the levels of diphosphopyridine nucleotide (DPN) in mouse liver. These transiently elevated DPN levels were maintained for a prolonged period when chlorpromazine was administered prior to the injection of nicotinamide (31). Burton, *et al.* (30), observed that nicotinamide, in concentrations which did not influence the duration of pentobarbital-induced hypnosis in mice, significantly extended the effect of chlorpromazine on pentobarbital sleeping time. In a subsequent study, Burton, *et al.* (32), found that "nontranquillizing" derivatives of phenothiazine (*e.g.*, thioperazine) were ineffective in maintaining elevated DPN levels. It would be interesting to determine whether a relationship exists between the synergistic activity of various phenothiazine derivatives and their ability to maintain nicotinamide-induced levels of DPN.

Inhibition of drug inactivating mechanisms is not held accountable for phenothiazine synergy by all investigators. Martin, *et al.* (138), observed that chlorpromazine and chlorpromazine sulfoxide enhanced the magnitude and prolonged the duration of the chronotropic action of epinephrine and norepinephrine in the spinal vagotomized cat. Although the factors in this phe-

nomenon were not elucidated, it was emphasized that chlorpromazine did not alter the metabolism of these catecholamines by either blood or liver.

Brodie, *et al.* (25) observed that mice which had just recovered from hypnosis induced by hexobarbital, reverted immediately to the hypnotic state when injected with chlorpromazine. In contrast, mice were not visibly affected by SKF 525-A or amine oxidase inhibitors of the hydrazine type administered after return of the righting reflex (123). These researchers concluded that SKF 525-A and the amine oxidase inhibitors prolong anesthesia by interferring with hexobarbital metabolism, whereas chlorpromazine increases the sensitivity of the central nervous system to depressant drugs. The precise nature of this "sensitization" remains to be elucidated; the extent to which it may involve the central adrenergic blocking activity of chlorpromazine (26) or the postulated alteration of the "trophotropic-ergotropic" equilibrium (24) has not received attention.

Several types of evidence have been cited in support of the concept that a reduction or stabilization of the permeability of certain biological membranes may constitute the primary action of chlorpromazine. Spirtes and Guth (190) observed that concentrations of chlorpromazine less than required to influence oxidative phosphorylation diminished the water imbibition of mitochondria induced by thyroxin and other swelling agents. Gey and Pletscher (87) have summarized other findings in support of this view and have suggested that a reduction of membrane permeability *in vivo* may account for their observation of interference by chlorpromazine with the metabolism of aromatic amino acids in rat brain. In a report on the influence of chlorpromazine on the activity of monoamine oxidase inhibitors and monoamine releasers in rat brain Schwartz, *et al.* (181), similarly concluded that the phenothiazine probably interferes with the permeation of monoamines or their precursors through brain membranes. There are no data which relate this concept to the possible mechanisms of phenothiazine synergy; nevertheless, it is conceivable that biotransformation processes may be impeded by reduced accessibility to intracellular sites of metabolism in the absence of direct inhibition of the enzymes involved.

Biochemical Mechanisms (SKF 525-A and Other Synergists).—The polyvalent nature of the synergistic activity of SKF 525-A has been attributed largely to an interference with the biotransformation of active drugs. This hypothesis is supported generally by the many reports which have established the prolongation of the action of various drugs by SKF 525-A, and specifically by those studies which have elucidated the effects of SKF 525-A on discrete metabolic pathways (17, 21, 42, 207).

SKF 525-A has been shown to inhibit the conversion of mephenesin to *o*-toloxylactic acid (135), the side-chain oxidation of hexobarbital, pentobarbital, and secobarbital (6, 48), the deamination of amphetamine, the formation of morphine glucosiduronide, the O-dealkylation of codeine (5, 48), the N-demethylation of aminopyrine, meperidine (48, 86), and the opiates (197), and the oxidation of phenylbutazone (29). Fouts and Brodie concluded that inhibition of drug-metabolizing enzyme systems present in liver microsomes (77) and in the soluble fraction of the liver cell (79) constituted a major factor in the synergistic activity of SKF 525-A.

The activity and/or toxicity of certain compounds is antagonized, rather than synergized, by SKF 525-A. This paradoxical effect has been explained on the basis of inhibition of enzyme systems required for the formation of active metabolites of the parent compound. For example, SKF 525-A diminished the prophylactic action of mepacrine against equine encephalomyelitis in mice (101, 202). This observation suggests, but does not prove, that SKF 525-A retards the conversion of mepacrine to an active antiviral metabolite. Organophosphates of the phosphorodiamidate and phosphorothionate classes are not toxic directly, but are converted to potent cholinesterase inhibitors by an oxidative reaction which, in mammals, is accomplished almost exclusively by liver microsomes (52). When added to mammalian liver preparations *in vitro*, SKF 525-A blocks the "activation" of schradan (tetramethylphosphordiamide anhydride), Guthion (dimethoxy ester of benzotriazine dithiophosphoric acid), and parathion (diethyl *p*-nitrophenyl phosphorothionate) (52, 151). SKF 525-A has been shown to be an effective antagonist of poisoning by schradan and Guthion, but not parathion in mice (155, 156). Presumably parathion possesses a greater affinity than the other organophosphates for the activating system *in vivo*, and therefore is not significantly altered by the presence of SKF 525-A (156). In summary, there have been cited multifaceted evidences that SKF 525-A influences enzymatic pathways involved in the detoxication of various drugs, and the conversion of certain latent species to biologically active molecules.

Interference with drug metabolism has been proposed as the basis for the synergistic activity

demonstrated with many compounds other than SKF 525-A. In the case of Lilly 18947 (77), iproniazid (78), JB 516 (123), captodiamine (65), chloramphenicol (57), and thyroxin (43), prolongation of hexobarbital sleeping time has been correlated with elevation of whole body concentrations of barbiturate and reduction of the rate of hexobarbital oxidation in an *in vitro* system. In studies of JB 516, N-ethyl-3-piperidyl benzilate and N-methyl-3-piperidyl diphenylcarbamate, Fujimoto, *et al.* (84), and Serrone and Fujimoto (182) related extension of hexobarbital hypnosis to whole body concentrations of the hypnotic, rates of disappearance from an isolated liver perfusion system, alteration of bromsulfalein retention, and inhibition of hexobarbital metabolism *in vitro*. Robison and Schueler (171) performed comparable studies with α-benzoylamine-β-(4-pyridyl)acrylic acid piperidide. Results of these investigations provided objective support of the proposed mechanism of action of the compounds in question. Such multidimensional analyses designed to elucidate fundamental synergic mechanisms are, unfortunately, the exception rather than the rule.

Data reported by several investigators suggest that the mechanism of action of SKF 525-A and certain other compounds which manifest synergic activity cannot be explained solely on the basis of retardation of drug metabolism. Several phenomena indicate that additional factors, not fully revealed at present, may also be operative. The phenomena include: (*a*) discriminative enhancement of certain properties of a drug, (*b*) reinstitution of a characteristic response by administration of the synergist after initial response to the active drug had terminated, (*c*) manifestation of a response by administration of a subthreshold dose of an active drug in combination with a synergist.

Corresponding specific observations involving SKF 525-A which do not conform totally to the "drug metabolism hypothesis," may be cited: (*a*) SKF 525-A prolonged hexobarbital-induced hypnosis without significantly altering the LD_{50} of the barbiturate (47) and enhanced the analgesic activity but not the respiratory depressant effect or LD_{50} of morphine (45). Similarly, the anticonvulsant activity and neurotoxicity of several central nervous system depressants were not uniformly increased by SKF 525-A (195). (*b*) Injection of SKF 525-A in the sciatic nerve-gastrocnemius muscle preparation of the chloralosed dog, after the muscle relaxant action of succinylcholine has ceased, resulted in a renewed and substantially increased curarizing effect (12, 18). These investigators proposed that the synergist displaced the muscle relaxant compound fixed in an inactive form at nonspecific receptor sites. A renewed and sharply defined depressor response was elicited by the administration of SKF 525-A after the blood pressure reducing effect of several different hypotensive drugs had largely dissipated in unanesthetized rats and dogs (90, 91). (*c*) These authors also observed that subthreshold doses of the hypotensive drugs evoked a significant decrease in blood pressure when injected after the administration of nondepressor doses of SKF 525-A. Such experiments lend support, albeit indirectly, to the concept of displacement of the active molecule from loci of nonspecific adsorption by the synergic agent.

Attention directed toward the role of biotransformation mechanisms in synergic phenomena, while most revealing, has tended to subjugate consideration of other basic aspects of the drug activity spectrum, *i.e.*, absorption, binding, cellular and subcellular distribution and localization, and elimination. A perspective of the kinetics of drug synergy must await investigations comparable to those reviewed in this section performed in conjunction with analyses of factors other than drug biotransformation which may influence the pharmacologic response. Parallel studies, utilizing both biodynamic and biochemical parameters, may clarify mechanisms of synergy in relationship to the total metabolic sojourn of the active drug.

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Chemical Identification of Defective Thyroid Preparations

By ALBERT D. WILLIAMS, LESTER MEISTER, and WARNER H. FLORSHEIM

A modification of the Leland and Foster method for estimation of the thyroxine-like fraction in thyroid materials is described. This chemical method can be used to identify commercial thyroid preparations which are defective by clinical evaluation or by biological assay. In contrast, no difference in the content of the thyroxine-like fraction of effective and defective thyroid samples could be shown by our use of Blau's method.

RECENTLY we have found that certain batches and brands of thyroid tablets were relatively ineffective in the treatment of hypothyroidism in agreement with other recent reports (1, 2). Except for a British Pharmacopoeia preparation, all material clinically studied had been standardized in accordance with U.S.P. requirements. Unusually large daily amounts of these defective preparations were needed by patients who nevertheless responded at least as well to the customary small daily doses of other lot numbers or brands of thyroid. Since it was apparent that the defective thyroid tablets contained excessive amounts of nonhormonal iodinated compounds, quantitation of the hormonally active fraction might be useful in the identification of "ineffective" thyroid preparations.

Leland and Foster (3) described a method for the determination of the thyroxine content in an alkaline hydrolysate of large quantities (1 Gm.) of desiccated thyroid. We have modified the Leland and Foster method by using the sealed-tube alkaline hydrolysis procedure of Kennedy (4) for which only small amounts (1/4 to 2 grains) of thyroid material are required. The iodinated compounds in the hydrolysate are extracted into *n*-butanol and a small portion of the butanol extract, after backwashing with alkali, is analyzed directly for iodine content using a procedure modified from Bodansky, *et al.* (5). The iodide-catalyzed decolorization of ceric ion by arsenite is stopped after a predetermined time by adding brucine sulfate as suggested by Grossman and Grossman (6) which produces a stable color for photometric measurement.

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PROCEDURE

Reagents.—Reagents 1–5 were prepared exactly as described by Bodansky, *et al.* (5); all were prepared in glass-distilled water. The reagents used were 1, sodium chromate (0.5%); 2, chloric acid (28%); 3, arsenious acid; 4, ceric ammonium sulfate; 5, potassium iodate standards; 6, brucine sulfate (1%) in water; 7, thiourea (0.2%) in water; 8, 2 *N* sodium hydroxide; and 9, 4 *N* sodium hydroxide saturated with *n*-butanol.

Total Thyroid Iodine.—A single thyroid tablet (1/4 to 2 grains)[1] or a 15 to 60-mg. sample of desiccated thyroid powder in a 25 × 150-mm. Pyrex tube was digested with 0.2 ml. of sodium chromate and 6 ml. of chloric acid. We modified the procedure of Bodansky, *et al.* (5), and digested the sample at 130° for 2 hours in an aluminum heating block with holes 4 cm. deep to accommodate the tubes. Different manufacturers formulate thyroid tablets with various and varying amounts of binders and diluents creating some problems in this first digestion step. Organic diluents in the larger tablets may reduce the chromate resulting in a potential loss of iodine. Additional chloric acid may be required for the complete digestion of such preparations. Certain inorganic binders will form an insoluble residue after digestion but this does not appear to interfere with subsequent steps in the procedure. The digested thyroid sample is quantitatively transferred to a volumetric flask (100 to 500 ml.) and diluted to volume with glass-distilled water. A small aliquot of the diluted sample (0.2 to 1.0 ml.) containing between 0.01 to 0.05 mcg. of iodide is pipetted in duplicate into 25 × 150-mm. test tubes. One milliliter of glass-distilled water (for reagent blanks)

[1] A 1-grain tablet is considered to contain 60 mg. of desiccated thyroid although we are aware that not all 1-grain thyroid tablets contain exactly this amount of thyroid powder.

and 1 ml. of each of the five working standards (0.01 to 0.06 mcg. of iodine per ml.) is pipetted in duplicate into similar tubes. Chloric acid (6 ml.) and sodium chromate (0.2 ml.) are added to the unknown samples, reagent blanks, and standards for digestion at 130° for 2 hours in the aluminum heating block. Glass-distilled water (10 ml.) and arsenious acid (2 ml.) are added to the tubes after digestion as described by Bodansky, *et al.* (5), and the tubes are placed in a 28° water bath. After temperature equilibration, 0.5 ml. of ceric ammonium sulfate is added in sequence at 30-second intervals to each tube, the tube contents rapidly mixed by swirling, and the tubes returned to the water bath. Exactly 25 minutes later, 0.5 ml. of brucine sulfate is added at 30-second intervals to each sample in sequence and mixed. Thus, the iodide-catalyzed ceric ammonium sulfate decolorization reaction is allowed to proceed for exactly 25 minutes in each tube before the reaction is stopped with brucine. The color intensity of each unknown sample, reagent blank, and standard is measured in a Klett photoelectric colorimeter using a No. 42 filter. The instrument is set to zero with water; the reagent blank readings are usually about 300 Klett units. The average reading of each unknown sample, and of each standard, is divided by the average blank reading. Since a linear relationship exists between the logarithm of each standard ratio and its concentration, a standard curve can be plotted on semilogarithmic paper as described by Kontaxis and Pickering (7). The iodide content of each unknown, determined from the standard curve, is multiplied by the appropriate dilution factor to obtain the iodine content in the original digested thyroid sample.

"Thyronine" Iodine.—Although Kennedy (4) used evacuated, sealed reaction tubes for the alkaline hydrolysis of thyroid, we have found it more convenient to place a single thyroid tablet (1/4 to 2 grains) or a 15 to 60-mg. sample of thyroid powder in a 20 × 185-mm. Thunberg tube. After adding 0.1 ml. (0.2 mg.) of thiourea and 12 ml. of 2 *N* sodium hydroxide to the sample, the tube is evacuated at the water pump and sealed (while pumping) by turning the lightly greased stopper. It is heated at 100° for 16 hours in an aluminum heating block. The lower 4 cm. of the Thunberg tube is heated directly by the block while the upper 14 cm. acts as an air-cooled reflux condensor. After hydrolysis, the tubes are cooled and the hydrolysate, with any insoluble residue, is transferred by pipet to 25 × 150-mm. screwcap Pyrex tubes. The Thunberg tube is rinsed with 12 ml. of *n*-butanol which is transferred to the alkaline hydrolysate in the screwcap tube. The tubes are tightly sealed with a small square of Parafilm under the screwcap and vigorously shaken for 1 minute. After phase separation by centrifugation, the butanol layer is removed to a volumetric flask and the aqueous phase is extracted a second time with an equal volume of *n*-butanol. After centrifugation, the second butanol extract is pooled with the first in the volumetric flask and diluted to volume with butanol. The diluted solution should contain about 0.1 to 0.2 mcg. of iodine per ml. Iodotyrosines are removed from a small portion of the diluted butanol extract by shaking with an equal volume of 4 *N* sodium hydroxide saturated with butanol and separating the phases by centrifugation. Aliquots of the alkali backwashed butanol extract (0.1 to 0.3 ml.), reagent blanks and iodide standards are pipetted into 25 × 150-mm. tubes for digestion with sodium chromate and chloric acid as described previously for the determination of total thyroid iodine. It is important to digest no more than 0.3 ml. of the butanol extract with the chloric acid reagent: *larger amounts of butanol react explosively when heated with chloric acid.* After digestion, iodine analysis is done as described previously for total thyroid iodine determination to obtain the butanol-extractable iodine content ("thyronine" iodine) in the alkaline hydrolysate of the thyroid preparation.

Clinical Evaluation and Bioassay of Thyroid Materials.—Many of the thyroid preparations mentioned in this report have been assayed for biological potency.[2] Methods used for bioassay, as well as the clinical evaluation of patient response to these thyroid preparations will be fully described in a forthcoming publication. The methods used for clinical assay purposes included most of the commonly accepted metameters such as the determination of the serum PBI and cholesterol levels, general clinical criteria (including body weight changes and subjective responses), and, in a few instances, the BMR. Primary emphasis was placed on the serum PBI values. Dosage levels were multiple whenever possible for both the standard and unknown thyroid preparations, the latter being given at both high and customary dosages.

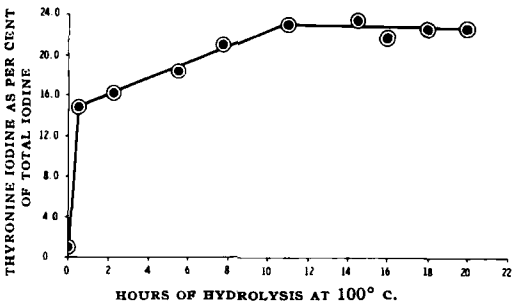

Fig. 1.—"Thyronine" iodine recovery from thyroid tablets subjected to varying periods of sealed tube alkaline hydrolysis. Points on the curve represent the average "thyronine" iodine as per cent of total iodine content for pairs of thyroid samples hydrolyzed for the indicated times.

RESULTS AND DISCUSSION

Identification of Samples.—With reference to the results to be discussed in this report, the thyroid tablets and powders are coded by number as they are received in the laboratory. Each code number refers to the lot number as received by us. A protocol has been filed with Dr. Lloyd C. Miller of the U.S.P. relating each code number to the brand and lot number of the material studied. In some cases, we have tested different lots of tablets or desiccated thyroid powders from the same pharmaceutical company.

Definition of "Thyronine" Iodine.—The term

[2] We wish to acknowledge with appreciation the cooperation of Wilson Laboratories, Chicago, Ill., and Armour Pharmaceutical Co., Kankakee, Ill., for bioassays of a number of thyroid preparations.

TABLE I.—"THYRONINE" IODINE RECOVERY

Sample	"Thyronine" Iodine, %	Total Iodine, %	"Thyronine" Iodine as % of Total Iodine
L-3,5-Diiodotyrosine	0.25	53.8	0.46
L-3,5-Diiodothyronine	21.8	47.8	45.6
L-3,3',5-Triiodothyronine	41.8	50.2	83.3
Sodium L-thyroxine powder	53.0	59.6	88.9
Sodium L-thyroxine powder	55.6	62.8	88.5
Sodium L-thyroxine tablets	43.8	50.0	87.6
Sodium L-thyroxine tablets	54.0	64.0	84.4
Sodium L-thyroxine tablets	44.7	48.0	93.1
Sodium L-thyroxine-I^{131}	9110 c/m[a]	10,240 c/m[b]	89.0
Sodium L-thyroxine-I^{131}	9580 c/m	10,590 c/m	90.5

[a] Total counts per minute recovered in the "Thyronine" iodine fraction. [b] Total counts per minute in the original sample corrected for 3% inorganic iodide-I^{131} counts as judged by filter paper chromatography.

"thyronine" iodine used in this report has special meaning. After alkaline hydrolysis of the thyroid material, the *n*-butanol soluble portion of the hydrolysate is backwashed with 4 *N* sodium hydroxide saturated with *n*-butanol to remove inorganic iodide and iodotyrosines. The alkali backwashed *n*-butanol extract of the hydrolysate contains chiefly thyroxine and triiodothyronine. The iodine content of this *n*-butanol fraction is referred to as "thyronine" iodine in this report although we recognize that this fraction may contain small amounts of other iodinated compounds.

Expression of "Thyronine" and Total Iodine Values.—"Thyronine" iodine and total iodine values are reported as per cent by weight of the material studied. In the case of thyroid tablets, it is assumed that a 1-grain thyroid tablet contains 60 mg. of desiccated thyroid material[1] and this weight is used to calculate per cent "thyronine" iodine or total iodine. In some of the tables, "thyronine" iodine is expressed as per cent of the total iodine content of the material studied. All values reported represent the mean of four or more individual determinations.

Optimal Time for Alkaline Hydrolysis.—The optimal time for alkaline hydrolysis was established by preparing a number of single 1 grain tablets of the same lot number for hydrolysis in sealed tubes. Pairs of tubes were removed at intervals from the heating block for "thyronine" iodine determinations. Points on the curve in Fig. 1 are the average of duplicates and represent "thyronine" iodine as per cent of the total iodine content of the tablet. The "thyronine" iodine recovery curve plateaus after 11 hours of hydrolysis with little change to 20 hours and resembles that reported by Leland and Foster (3). An overnight hydrolysis is convenient and all "thyronine" iodine values in this report are based on a 16 hour hydrolysis period.

Total Iodine Recovery Studies.—To evaluate our method for total iodine analysis of desiccated thyroid powders, thyroglobulin extracts, iodinated casein and L-sodium thyroxine, we compared our total iodine values with the manufacturer's reported total iodine content in 21 different samples. The mean recovery by our method was 98.5 ± 3.3 (s.d.) % of the manufacturer's indicated total iodine value (range 92.3 to 107.7%).

Thyroxine Iodine Recovery Studies.—Sodium L-thyroxine powder and sodium L-thyroxine tablets were analyzed for total and "thyronine" iodine by our method. Theoretically "thyronine" iodine represents thyroxine iodine in these samples and should correspond to the total iodine content. Results are listed in Table I for "thyronine" and total iodine content of thyroxine and other iodinated compounds. Also included in Table I are radioactive thyroxine recovery studies. Mixtures of thyroxine-I^{131} and non-radioactive thyroxine were hydrolyzed in sealed tubes and extracted with *n*-butanol for radioactivity measurement. The total counts per minute in the original thyroxine-I^{131} samples in Table I were corrected for a 3% contamination by inorganic-I^{131} as judged by paper chromatography.

TABLE II.—REPRODUCIBILITY OF REPEATED DETERMINATIONS ON THE SAME SAMPLE

	1 Grain Thyroid Tablets	Undiluted Thyroid Powder
Total Iodine; %		
Mean	0.213	0.747
Std. Dev.	0.005	0.013
No. of Samples	17	14
"Thyronine" Iodine; %		
Mean	0.0491	0.181
Std. Dev.	0.0025	0.006
No. of Samples	18	14

The mean recovery of iodine in the "thyronine" fraction in seven thyroxine samples (Table I) is 88.9 ± 2.7 (s.d.)% of the total iodine content found. This recovery is somewhat better than that reported by Leland and Foster (3) who observed a 25% loss of pure thyroxine in their alkaline hydrolysis method. Blau (8), in a study of the alkaline hydrolysis of thyroid, recovered 97 and 90% of thyroxine iodine when pure samples of thyroxine were boiled in barium hydroxide or in sodium hydroxide, respectively. Kroc, *et al.* (9), in control studies with pure thyroxine, recovered 88 to 90% of the theoretical iodine using Blau's method. In contrast to the recoveries of Leland and Foster, our more satisfactory recoveries may be due to the use of partially evacuated sealed tubes and to the protective effect of the thiourea used during alkaline hydrolysis.

Recovery of iodine in the "thyronine" iodine fraction from samples of diiodotyrosine, diiodothyronine, and triiodothyronine was determined by sealed tube alkaline hydrolysis and *n*-butanol extraction. It is evident from the results in Table I that only a very small fraction of diiodotyrosine iodine remains in the backwashed *n*-butanol extract but about 45% of any diiodothyronine iodine, if present, will be

found in this fraction. Recovery of triiodothyronine iodine (83%) is similar to that reported by Kroc, *et al.* (9).

Reproducibility of the Method.—The "thyronine" and total iodine content of one of our samples, a 1 grain thyroid tablet of known clinical potency, has been determined on many individual tablets in a number of different experiments during the past year. A summary of these results (Table II) shows good reproducibility of the method considering that some individual variation in both total and "thyronine" iodine content in the tablets of this particular lot number may exist. Included in Table II are results for the total and "thyronine" iodine content in fourteen 20-mg. samples of an undiluted thyroid powder analyzed in a single experiment which also show good reproducibility.

Effect of Lactose on "Thyronine" Iodine Recovery.—Lactose, used as a diluent in some desiccated thyroid preparations to reduce the total organic iodine content to U.S.P. or B.P. specifications, was shown by Doery (10) to interfere with the analysis of thyroxine in thyroid. Addition of lactose to desiccated thyroid powder increased the thyroxine iodine value when measured by the 1936 Addendum to the B.P. (1932) method for thyroid. Johnson and Smith (11) using the B.P. (1958) method for thyroid assay confirmed Doery's observations. Doery (10) pointed out that the sodium hydroxide was partially neutralized by organic acids formed from lactose during hydrolysis. Under these less effective hydrolytic conditions, incompletely digested protein (containing non-thyroxine iodine) was adsorbed to the flocculent precipitate which formed after acdification resulting in increased thyroxine iodine values. Doery (10) also measured the recovery of thyroxine from thyroid by the butanol extraction method of Leland and Foster (3) and observed a significant (18 to 29%) loss of thyroxine when thyroid powder was hydrolyzed with sodium hydroxide in the presence of added lactose.

The results of Doery prompted us to study the influence of added lactose on "thyronine" iodine recovery by our method from thyroid powders and from sodium L-thyroxine. The results of our experiments are shown in Table III. In this table, samples 16, 17, and 23 are undiluted, desiccated thyroid powders containing from 0.65 to 0.80% total iodine. Sample 28 is sodium L-thyroxine. The samples were hydrolyzed in the absence of and also in the presence of 20 and 60 mg. of added lactose. The butanol extracts were then analyzed for "thyronine" iodine. The reduction in the "thyronine" iodine value by lactose is relatively small, and the differences we find in the "thyronine" iodine content of effective and defective thyroid preparations cannot be ascribed to the presence of lactose in the defective material. In our procedure, the ratio of sodium hydroxide to thyroid material is greater than that used by Leland and Foster (3). The increased sodium hydroxide content and the use of thiourea in the alkaline hydrolysis step of our procedure may decrease the lactose effect described by others (10, 11).

Evaluation of Blau's Method for Thyroxine Iodine in Thyroid.—Blau (8) described a method for the determination of thyroxine in thyroid materials in which barium hydroxide hydrolysates were acidified prior to extraction of thyroxine into butanol. After backwashing with Blau's reagent, the butanol solu-

TABLE III.—EFFECT OF ADDED LACTOSE ON RECOVERY OF "THYRONINE" IODINE

Sample No.	Sample Wt., mg.	%"Thyronine" iodine in sample with: No Lactose	Lactose, 20 mg.	Lactose, 60 mg.	% Recovery of "Thyronine" iodine in presence of: Lactose, 20 mg.	Lactose, 60 mg.
16	15	0.209	0.196	0.197	93.8	94.3
17	20	0.109	0.104	0.099	95.4	90.8
23	30	0.177	0.163	0.162	92.1	91.5
28[a]	20	53.0	49.6	...	93.6	...

[a] Sodium L-thyroxine powder.

TABLE IV.—COMPARISON OF BLAU'S METHOD WITH THE PRESENT METHOD FOR "THYRONINE" IODINE

Sample No.	Total Iodine, %	"Thyronine" Iodine, % Present Method	Blau's Method	"Thyronine" Iodine as % of Total Iodine: Present Method	Blau's Method	Ratio Blau's Method / Present Method
		Ineffective Thyroid Preparations				
17	0.650	0.109	0.157	16.8	24.2	1.44
9	0.205	0.0295	0.0435	14.4	21.2	1.47
6	0.232	0.0317	0.0480	13.7	20.7	1.51
36	0.208	0.0270	0.0605	13.0	29.1	2.24
57	0.565	0.0725	0.143	12.8	25.3	1.98
42	0.213	0.0270	0.0502	12.7	23.6	1.86
37	0.220	0.0275	0.0390	12.5	17.7	1.42
		Effective Thyroid Preparations				
16	0.800	0.209	0.209	26.1	26.1	1.00
24	0.205	0.0488	0.0522	23.8	25.5	1.07
13	0.242	0.0566	0.0515	23.4	21.3	0.91
44	0.213	0.0491	0.0442	23.1	20.8	0.90
8	0.203	0.0460	0.0605	22.7	29.8	1.31
56	0.795	0.168	0.216	21.1	27.2	1.29
55	0.881	0.187	0.221	21.2	25.1	1.18
58	0.210	0.0443	0.0442	21.1	21.0	0.99

tion was analyzed for iodine. Blau stated that results by his method agreed with those obtained by the B.P. acid precipitation method for samples of commercial thyroid powder although neither his nor the B.P. method gave consistent results with thyroid tablets. He felt that the discrepancies observed in the analysis of thyroid tablets could be attributed to adsorption of iodinated compounds to the excipients used in formulating the tablets. Doery (10) suggested that acidification of a mixture of soluble and insoluble products in the alkaline hydrolysate favored adsorption of non-thyroxine iodine to the thyroxine precipitate.

We determined the "thyronine" iodine content in a number of commercial thyroid powders and tablets using the method of Blau (8) and these results are compared in Table IV with results obtained on the same preparations by our method. The first seven samples in Table IV were defective, and the last eight samples were effective preparations when tested biologically or clinically.

The data in Table IV clearly indicate that, by our method, the results for "thyronine" iodine (calculated as per cent of total iodine) sharply discriminate between effective and ineffective thyroid preparations. In contrast, using Blau's method for the measurement of "thyronine" iodine content of thyroid materials, we were unable to detect any significant difference between the effective and ineffective preparations. It is of particular interest that the "thyronine" iodine content of the effective preparations is comparable by both methods. The ineffective preparations, however, have "thyronine" iodine values by the Blau method which, on the average, exceed by a factor of 1.7 the corresponding results by our method.

Since all preparations in Table IV complied with or exceeded U.S.P. specifications, the high values by Blau's method for "thyronine" iodine in ineffective material are due to nonhormonal iodinated residues in the alkaline hydrolysate, possibly as incompletely hydrolyzed polypeptides. Such residues may be more readily soluble in butanol after acidification of the hydrolysate and are inefficiently removed when the butanol extract is backwashed with sodium hydroxide. The iodine content of the resulting

TABLE V.—SUMMARY OF RESULTS ON EFFECTIVE THYROID TABLETS AND POWDERS

Sample No.	Total Iodine[a], %	"Thyronine" Iodine[a], %	"Thyronine" Iodine as % of Total Iodine	Effective by: Clinical Assay	Effective by: Bioassay
40	0.220	0.0463	21.0	x	x
56	0.795	0.168	21.1	...	x
65	0.227	0.0478	21.1	...	...
58	0.210	0.0443	21.1	...	...
55	0.881	0.187	21.2	...	x
10	0.230	0.0491	21.3	...	...
51	0.192	0.0413	21.5	...	...
61	0.228	0.0496	21.8	x	...
59	0.217	0.0480	22.1	x	...
22	0.212	0.0471	22.2	x	...
33	0.222	0.0496	22.3	x	...
8	0.203	0.0460	22.7	x	...
47	0.855	0.194	22.7	...	...
77	0.233	0.0532	22.8	...	...
44	0.213	0.0491	23.1	x	...
29	0.263	0.0608	23.1	x	x
2	0.242	0.0558	23.1	...	...
35	0.203	0.0471	23.2	...	...
11	0.223	0.0518	23.2	...	...
52	0.237	0.0552	23.3	...	...
13	0.242	0.0566	23.4	x	x
12	0.213	0.0506	23.8	x	...
34	0.202	0.0481	23.8	x	...
24	0.205	0.0488	23.8	...	x
69	0.218	0.0525	24.1	x	x
4	0.208	0.0511	24.6	...	...
65	0.212	0.0521	24.6	x	x
14	0.223	0.0558	25.0	x	x
23	0.708	0.177	25.0	...	...
30	0.218	0.0551	25.3	x	x
5	0.200	0.0510	25.5	...	...
19	0.205	0.0523	25.5	...	...
64	0.218	0.0556	25.5	x	...
49	0.708	0.181	25.6	...	...
18	0.212	0.0550	25.9	...	...
53	0.203	0.0530	26.1	...	...
16	0.800	0.209	26.1	...	x
72	0.585	0.154	26.3	...	...
15	0.218	0.0600	27.5	x	...
32	0.333	0.0930	27.9	x	...
73	0.357	0.100	28.0	...	x
75	0.555	0.161	29.0	...	x
76	0.484	0.142	29.3	...	x
31	0.208	0.0616	29.6	x	x

[a] In the case of thyroid tablets, this per cent is based on an assumed weight of 60 mg. of desiccated thyroid powder per 1-grain tablet. (See footnote 1 in text.)

TABLE VI.—SUMMARY OF RESULTS ON DEFECTIVE THYROID TABLETS AND POWDERS

Sample No.	Total Iodine[a], %	"Thyronine" Iodine[a], %	"Thyronine" Iodine as % of Total Iodine	Defective by: Clinical Assay	Defective by: Bioassay
54	0.245	0.0280	11.4	x	x
37	0.220	0.0275	12.5	x	
39	0.240	0.0303	12.6	x	
42	0.213	0.0270	12.7	x	x
57	0.565	0.0725	12.8		x
36	0.208	0.0270	13.0	x	
62	0.215	0.0282	13.1		
41	0.215	0.0283	13.2	x	x
6	0.232	0.0317	13.7	x	x
3	0.208	0.0293	14.1		
9	0.205	0.0295	14.4	x	x
74	0.247	0.0362	14.6		x
7	0.210	0.0308	14.7	x	x
50	0.595	0.0905	15.2		x
20	0.225	0.0347	15.4	x	
79	0.575	0.0945	16.4		
60	0.203	0.0335	16.5		
17	0.650	0.109	16.8		x

[a] In the case of thyroid tablets, this per cent is based on an assumed weight of 60 mg. of desiccated thyroid powder per 1-grain tablet. (See footnote 1 in text.)

butanol extract, therefore, is due not only to "thyronine" iodine but to an excess of nonhormonal iodinated materials. It is possible that the longer period of sodium hydroxide hydrolysis used in our method and the direct butanol extraction of the alkaline hydrolysate results in an extract which, after backwashing with alkali, has an iodine content more nearly reflecting the true hormonal iodine content of the thyroid material. The effective thyroid materials containing less non-thyroxine iodinated substances would be expected to yield "thyronine" iodine values by Blau's method which are comparable to or only slightly higher than the results by our method.

Results with Present Method.—*Effective Thyroid.* The analytical results, by our method, for all thyroid tablets and desiccated thyroid or thyroglobulin powders which we studied and consider to be effective are presented in Table V. Of the 44 samples in this table, 26 are known to be clinically or biologically effective. The remaining 18 samples in this group, while not tested directly by us, were submitted to our laboratory as "good" thyroid and no unsatisfactory biological or clinical results with any of these preparations have been reported to us. In Table V, the per cent of total and "thyronine" iodine content of each sample identified by our code number is listed. Additionally, a value for the "thyronine" iodine as per cent of the total iodine content of the sample is given along with an indication of which materials studied were clinically or biologically adequate. The mean "thyronine" iodine as per cent of the total iodine content of the effective thyroid preparations in Table V is 24.2 ± 2.35 (s.d.).

Defective Thyroid.—The analytical values for "thyronine" and total iodine in a group of defective thyroid preparations are given in Table VI. Fourteen of the samples in this table have been shown to be either clinically or biologically defective. The remaining four samples, not tested clinically or biologically, are considered as unsatisfactory preparations since "thyronine" iodine expressed as per cent of total iodine content of these samples falls within the range of known defective thyroid material. One of these samples, No. 62, is known to have been formulated with desiccated thyroid powder from the same source used for four other samples in Table VI which were shown to be clinically or biologically defective. The thyroid tablets in Table VI were sold as U.S.P. thyroid and all except one, No. 39 (which had a slight excess of total iodine), met the requirements of the U.S.P. in terms of total organic iodine content. The mean "thyronine" iodine as per cent of total iodine in the defective thyroid group is 14.1 ± 1.55 (s.d.).

SUMMARY AND CONCLUSIONS

A summary of all thyroid preparations studied is given in Table VII. A highly significant difference ($p = < 0.001$) exists between the mean "thyronine"

TABLE VII.—SUMMARY OF ALL THYROID SAMPLES STUDIED

	Effective Samples	Defective Samples
"Thyronine" iodine as per cent of total iodine (Mean):	24.2	14.1
Standard error	±0.35	±0.37
Number of samples tested chemically	44	18
Range of values	21.0–29.6	11.4–16.8
Number of samples tested clinically or biologically or both	26	14
Number effective by clinical testing	19	0
Number effective by biological testing	15	0
Number defective by clinical testing	0	10
Number defective by biological testing	0	10

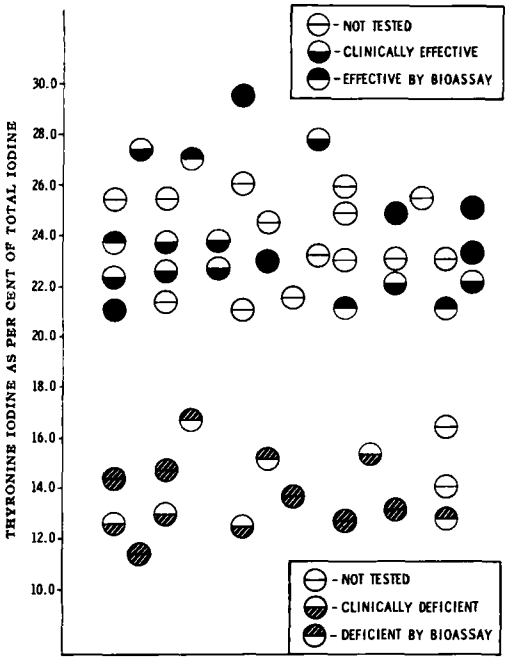

Fig. 2.—Scattergram of the "thyronine" iodine content as per cent of total iodine for individual thyroid preparations. Each point on the graph represents a different thyroid preparation and the symbols used indicate the thyroid preparations which tested clinically or biologically effective or deficient.

iodine per cent of total iodine in the effective and the defective samples with no overlapping of values between the two groups. Twenty-six of the effective and 14 of the defective preparations were biologically or clinically evaluated; some samples in each group were tested by both methods. In the effective group, 19 samples were clinically effective, 15 were biologically effective, and none were defective. In contrast, the defective group contained 10 samples which were clinically and 10 which were biologically defective, but none that were biologically or clinically effective.

In Fig. 2, the results of our chemical evaluation of some of the thyroid preparations studied are presented graphically together with an indication of those particular samples which were examined by clinical or bioassay methods.

In conclusion, we have described a method for the chemical analysis of the "thyronine" iodine content of commercial thyroid material. The "thyronine" iodine content expressed as per cent of the total iodine content of the thyroid samples correlates well with clinical or biological evaluation of the thyroid preparations.

ADDENDUM

The thyroid preparations listed in the table below have been examined for their content of triiodothyronine and thyroxine iodine in the laboratories of the Physiology and Hormones Section of the Canadian Food and Drug Directorate through the kind collaboration of Dr. N. R. Stephenson and Mr. W. F. Devlin using methods previously published by these authors [*J. Pharm. Pharmacol.*, **14**, 597 (1962)]. In the table, the values obtained by Devlin and Stephenson are compared with our "thyronine" iodine values expressed as per cent of total iodine, revealing a substantial degree of correlation.

COMPARISON OF THYROXINE AND TRIIODOTHYRONINE IODINE CONTENT WITH "THYRONINE" IODINE CONTENT OF EFFECTIVE AND DEFECTIVE THYROID PREPARATIONS

Sample No.	Triiodothyronine Iodine Plus Thyroxine Iodine as % of Total Iodine[a]	"Thyronine" Iodine as % of Total Iodine[b]
	Effective Preparations	
16	23.4	26.1
18	25.0	25.9
24	22.6	23.8
73	28.6	28.0
75	24.4	29.0
76	23.5	29.3
	Defective Preparations	
17	10.8	16.8
50	13.4	15.2
54	13.3	11.4

[a] Reported by Devlin and Stephenson (unpublished observations). [b] See Tables V and VI of this paper.

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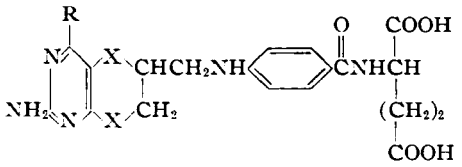
Analogs of Tetrahydrofolic Acid VII

Synthesis of N-[1-(2-Amino-4-hydroxy-6-methyl-5-pyrimidyl)-3-propyl]-*p*-aminobenzoyl-L-glutamic Acid, An Inhibitor of Folic Reductase

By B. R. BAKER and C. E. MORREAL

The title compound (XI) has been synthesized in a six-step sequence using ethyl acetoacetate, acrolein, guanidine, and *p*-aminobenzoyl-L-glutamic acid. This compound inhibits folic reductase and binds to the enzyme better than the substrate, folic acid. It also inhibits dihydrofolic reductase.

FIFTEEN ENZYMES UTILIZING folic acid, tetrahydrofolic acid (I), or derivatives of tetrahydrofolic acid are known (2–4). A number of these enzymes are inhibited (5–8) by aminopterin (4-amino-4-deoxyfolic acid), but nearly as many are not (7, 9–11). In contrast, 5,6,7,8-tetrahydroaminopterin (II) can inhibit some of the enzymes not inhibited by aminopterin (11, 12). 5,8-Dideaza-5,6,7,8-tetrahydroaminopterin (IV) has been recently synthesized (13) and found to have inhibitory properties similar to tetrahydroaminopterin (13, 14). In addition, 5,8-dideaza-5,6,7,8-tetrahydrofolic acid (III) (15) has been found to bind to folic reductase eight times stronger than the substrate, folic acid (16).



I. R = OH, X = —NH—
II. R = NH_2, X = —NH—
III. R = OH, X = —CH_2—
IV. R = NH_2, X = —CH_2—

The folic cofactor area should be a prime target for utilization of recent developments in nonclassical antimetabolite theory (17–19), since larger differential effects on inhibition of these enzymes might be obtained by the bulk principle of specificity (17), the exo-alkylating irreversible inhibition phenomenon (18), and the bridge principle of specificity (19). In order to use these three corollaries of nonclassical antimetabolite theory, it would be advisable to have an inhibitor that can be made by a relatively short sequence and the sequence should be one that lends itself to the placing of substituents in a variety of positions. Synthesis of compound XI, which satisfies both the inhibitor and synthetic requirements, is the subject of this paper.

Although the synthesis of ethyl 2-acetylglutaraldehydate (V) by Michael addition of ethyl acetoacetate to acrolein has been described by Moe and Warner (20), but no yield recorded, their procedure gave 5–20% yields in this laboratory. After considerable experimentation, consistent yields were obtained when it was finally realized that the reaction of ethyl acetoacetate with acrolein was so rapid that a 2:1 adduct between acrolein and the keto ester was being obtained before an individual drop could be adequately mixed. By addition of an ethanolic solution of acrolein to the vortex of a stirred ethanol solution of a 30% excess of ethyl acetoacetate containing a trace of sodium ethoxide, consistent yields of about 47% of V were obtained. The aldehyde function of V was then selectively converted to a diethyl acetal (VI)[1] in 56% of yield by reaction of V with boiling ethanol containing ammonium chloride, under which conditions the ketone function was not converted to an enol ether.

Reaction of VI with guanidine hydrochloride and sodium methoxide in boiling absolute ethanol gave the crystalline pyrimidine VII in 76% yield; guanidine carbonate was equally effective. In order to complete the synthesis of XI *via* the anil X, it was considered necessary to block the 2-amino group of VII, before the aldehyde function was unblocked in order to avoid polymerization of the amino aldehyde (13, 15, 21). Acetylation of VII with hot acetic anhydride (13, 15, 21) gave small and variable yields of the crystalline acetamido acetal, VIII; the oily by-products appeared to contain *O*-acetate bands in the infrared, suggesting that some acetolysis of the acetal function was taking place in the mildly acidic medium. As a result of this apparent side reaction, acetylation was run with acetic anhydride in pyridine at 85° when consistent yields of crystalline VIII in the order of 57% were obtained.

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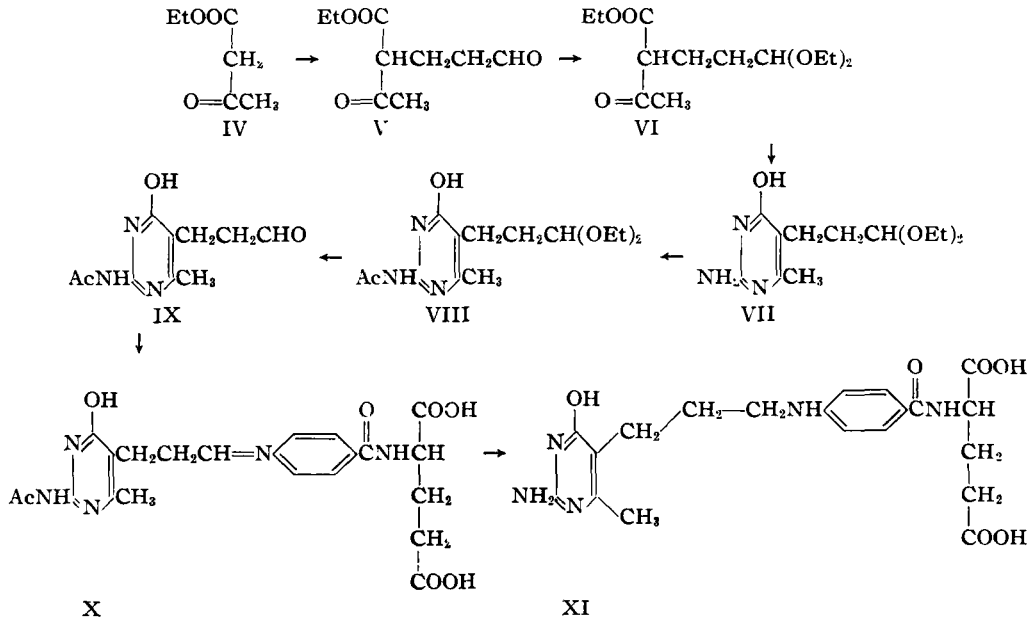
[1] Attempts to prepare VI by condensation of ethyl acetoacetate with β-chloropropionaldehyde diethyl acetal in absolute ethanolic sodium ethoxide with or without the presence of sodium iodide were unsuccessful. The reaction was slow, the products boiled over a wide range and examination of various fractions by analysis and infrared spectra did not give data compatible with structure VI.



Hydrolysis of the acetal function of VIII with 90% formic acid (13, 15, 21) gave variable yields of the crystalline aldehyde IX; unfortunately, the best yields were obtained on quantities of less than 1 Gm. of VIII. However, short boiling in water was sufficient to hydrolyze the acetal group of VIII to IX which was readily purified in consistent yields of 45–50%.

When a solution of the aldehyde IX and *p*-aminobenzoyl-L-glutamic acid in ethanol was refluxed for 1 hour, an amorphous precipitate separated from solution that resisted purification and gave poor analytical values for the supposed anil, X. In addition, the maximum amount of *p*-aminobenzoyl-L-glutamic acid, as determined quantitatively by the Bratton-Marshall reaction (22), that could be obtained on mild acid hydrolysis was 37–44 mole%; the analytical figures and the Bratton-Marshall values indicated that some de-N-acetylation of IX or X or both had occurred in the absolute alcohol, followed by the formation of higher condensation products. Although the anil was highly impure, this material did serve for working out suitable conditions for reduction and de-N-acetylation of X to the desired folic acid analog, XI.

The reduction of crude X with sodium borohydride was followed by a combination of change in ultraviolet spectrum at pH 1 and the amount of Bratton-Marshall dye obtained after short acid hydrolysis. The reduced product XI, had a peak at 222 mμ[2] that was absent in the anil, X, but present in *p*-aminobenzoyl-L-glutamic acid at 224 mμ with lower extinction coefficient; thus the reaction could be followed by the increase in extinction. The reduction of X in aqueous sodium bicarbonate with a large excess of sodium borohydride was complete in 1 hour at room temperature; the reaction was followed by periodic removal of aliquots, short hydrolysis in 1.5 *N* acid to remove the N-acetyl group, then measurement of the extinction coefficient at 222 mμ in 0.1 *N* acid. In this way the first sample of pure XI was obtained in about 15% yield.

Since the formation of the anil X in absolute ethanol led to side-reactions involving alcoholysis of the N-acetyl group, the anil was prepared *in situ* by reaction of IX with *p*-aminobenzoyl-L-glutamic acid in dimethylformamide; without isolation, the anil was reduced with sodium borohydride after the addition of methanol. In this way, XI was prepared in 44% yield (based on IX) containing about 5% of Bratton-Marshall positive material. Final purification afforded 21% of XI (based on IX) of pure product giving a negative Bratton-Marshall test.

The folic acid analog, XI, inhibited folic reductase (16) and had a $K_i = 2 \times 10^{-6}$, which is one-fifth the K_m value of folic acid. This compound also inhibited the dihydrofolic reductase from amethopterin-resistant Ehrlich ascites; an 0.2 m*M* concentration of XI gave a 66% inhibition of the enzymatic reaction run in the presence of 0.02 m*M* dihydrofolic acid (23). Since this compound is constructed from ethyl acetoacetate, acrolein, guanidine, *p*-aminobenzoic acid, and L-glutamic acid, it is obvious that a variety of substituted derivatives of XI can be made by modifying the five components or by transformations of VII; in this way, compounds could be obtained that—by use of non-classical antimetabolite theory (17–19)—might selectively inhibit some of the 15 enzymes in the folic acid cofactor area. In addition, considerable information could be obtained about the relative binding and conformational requirements of these substrates when complexed to their respective enzymes (14). Such a program is continuing in these laboratories.

EXPERIMENTAL[3]

Ethyl 2-Acetylglutaraldehydate (V).—To a vigor-

[2] It could be anticipated that the ultraviolet spectrum of XI and III (15) would be similar, since their chromophores are identical.

[3] Melting points were determined on a Kofler Heizbank and are corrected. Ultraviolet spectra were obtained on a Cary 11 recording spectrophotometer and infrared spectra on a Perkin-Elmer 137B recording spectrophotometer.

ously stirred solution of 130 Gm. (1 mole) of ethyl acetoacetate and 0.1 Gm. of sodium methoxide in 350 ml. of absolute ethanol was added dropwise to the vortex, a solution of 46.4 Gm. (0.83 mole) of acrolein in 100 ml. of absolute ethanol; the addition time was 70 minutes, the temperature was maintained at 10–13° by adequate cooling, and the solutions were protected from moisture. After standing for an additional 30 minutes without cooling, the solution was neutralized with acetic acid, then spin-evaporated to a syrup *in vacuo*. A solution of the syrup in 300 ml. of chloroform was washed with water, dried with magnesium sulfate and evaporated *in vacuo*. Distillation of the residue gave 66.2 Gm. (47%) of a colorless oil, b.p. 89–101° (0.5 mm.), that was suitable for the next step. Redistillation of a similar preparation gave pure material, b.p. 99–101° (0.5 mm.); $\lambda_{max.}^{film}$ 3.68 (aldehyde CH); 5.79 (aldehyde and ester C=O); 5.88 (ketone C=O); 6.12 (weak enolic C=C); 8.09, 8.20 μ (ester C—O—C).

Ethyl 2-Acetylglutaraldehydate Diethyl Acetal (VI).—To a solution of 66.2 Gm. (0.36 mole) of V in 450 ml. of absolute ethanol was added 2 Gm. of ammonium chloride. After being refluxed for 2 hours, the mixture was spin-evaporated to a syrup *in vacuo*. The residue was partitioned between 300 ml. of methylene chloride and 50 ml. of water. Washed again with 50 ml. of water and dried with magnesium sulfate, the organic solution was evaporated *in vacuo* and the residue distilled. After a forerun of 7.7 Gm. of unchanged V, the product distilled as a colorless oil, b.p. 107–124° (0.3 mm.) that was suitable for the next step; yield, 46.3 Gm. (50%). Redistillation gave the pure product, b.p. 110–112° (0.2 mm.); $\lambda_{max.}^{film}$ 5.77 (ester C=O); 5.84 (ketone C=O); 6.12 (weak enolic C=C); 8.09 (ester C—O—C); 9.45 μ (ether C—O—C). When the reaction was run for 4.5 hours, the yield was increased to 56%.

Anal.—Calcd. for $C_{13}H_{24}O_5$: C, 60.1; H, 9.64. Found: C, 60.0; H, 9.70.

β-(2-Amino-4-hydroxy-6-methyl-5-pyrimidyl)-propionaldehyde Diethyl Acetal (VII).—To a mixture of 1.52 Gm. (0.016 mole) of guanidine hydrochloride and 0.86 Gm. (0.016 mole) of sodium methoxide in 40 ml. of absolute ethanol was added 4.16 Gm. (0.016 mole) of VI. After being refluxed for 19 hours, the solvent was spin-evaporated *in vacuo*. The residue was partitioned between 50 ml. of chloroform and 30 ml. of water. The separated chloroform layer was dried with magnesium sulfate, then evaporated to dryness *in vacuo*. Recrystallization of the residue from ethyl acetate gave 3.1 Gm. (76%) of product, m.p. 176–177°, that was suitable for the next step. Further recrystallization from ethyl acetate afforded white crystals, m.p. 179–180°; $\lambda_{max.}^{KBr}$ 2.93, 3.14 (OH, NH); 6.10, 6.29 (NH, pyrimidine ring); 9.45 μ (ether C—O—C).

Anal.—Calcd for $C_{12}H_{21}N_3O_3$: C, 56.6; H, 8.31; N, 16.5. Found: C, 56.5; H, 8.10; N, 16.7.

β-(2-Acetamido-4-hydroxy-6-methyl-5-pyrimidyl)propionaldehyde Diethyl Acetal (VIII).—A solution of 15 Gm. of VII in 60 ml. of reagent pyridine and 40 ml. of acetic anhydride was heated in a bath at 80–90° for 1 hour protected from moisture. The solution was spin-evaporated to a syrup *in vacuo;* the evaporation was then repeated after the addition of 60 ml. of toluene. Recrystallization of the residue from ethyl acetate gave 10 Gm. (57%) of product, m.p. 148–150°. Further recrystallization from ethyl acetate afforded white crystals, m.p. 150°; $\lambda_{max.}^{KBr}$ 3.15 (NH); 6.05 (amide I); 6.28, 6.38 (NH and pyrimidine ring); 9.44, 9.66 μ (ether C—O—C).

Anal.—Calcd. for $C_{14}H_{23}N_3O_4$: C, 56.6; H, 7.81; N, 14.1. Found: C, 56.8; H, 7.94; N, 14.3.

β-(2-Acetamido-4-hydroxy-6-methyl-5-pyrimidyl)propionaldehyde (IX).—A solution of 1.00 Gm. (4.5 mmoles) of VIII in 50 ml. of water was refluxed for 1 hour, then spin-evaporated to dryness *in vacuo*. Two recrystallizations from ethyl acetate gave 0.34 Gm. (45%) of pure IX, m.p. 158–160°; $\lambda_{max.}^{pH\ 1}$ 228 (ϵ 8880), 270 mμ (ϵ 8270); $\lambda_{max.}^{pH\ 8.4}$ 270 mμ (ϵ 5350); $\lambda_{max.}^{pH\ 13}$ 278 mμ (ϵ 10,500); $\lambda_{max.}^{KBr}$ 3.16 (OH, NH); 3.65 (aldehyde CH); 5.87 (aldehyde C=O); 6.16 (amide I and pyrimidine ring); 6.74 (NH and pyrimidine ring); no ether C—O—C near 9.5 μ.

Anal.—Calcd. for $C_{10}H_{13}N_3O_3$: C, 53.8; H, 5.87; N, 18.8. Found C, 53.6; H, 5.95; N, 18.6.

N-[1-(2-Amino-4-hydroxy-6-methyl-5-pyrimidyl)-3-propyl]-p-aminobenzoyl-L-glutamic Acid (XI).—A solution of 100 mg. (0.5 mmoles) of IX and 120 mg. (0.5 mmoles) of *p*-aminobenzoyl-L-glutamic acid in 2 ml. of dimethylformamide was allowed to stand for 10 minutes, then diluted with 15 ml. of reagent methanol. The solution was then treated with 0.20 Gm. of sodium borohydride portionwise with stirring over a period of 30 minutes, then stirred overnight at room temperature. After the addition of 5 ml. of 0.1 *N* sodium hydroxide, the solution was spin-evaporated *in vacuo* to 5 ml., then acidified to pH 5 with 3 *N* hydrochloric acid. After being chilled for several hours, the mixture was centrifuged and the white solid washed successively with water (2 × 5 ml.), absolute alcohol (2 × 5 ml.) and dry ether (5 ml.); yield, 84 mg. (44%) that contained 5% of Bratton-Marshall positive material calculated as the anil (X). Recrystallization from dimethylformamide by addition of water gave 40 mg. (21%), m.p. >250°, of pure material, as determined by its ultraviolet spectra and negative Bratton-Marshall test. A similar preparation was analyzed and had $\lambda_{max.}^{pH\ 1}$ 222 (ϵ 27,800), 270 (ϵ 19,200), and 303 mμ (ϵ 11,400); $\lambda_{max.}^{pH\ 8.4}$ 295 mμ (ϵ 15,100); $\lambda_{max.}^{pH\ 13}$ 284 mμ (ϵ 20,500).

Anal.—Calcd. for $C_{20}H_{23}N_5O_6$: C, 55.8; H, 5.85; N, 16.2. Found: C, 56.2; H, 5.66; N, 16.5.

The Bratton-Marshall test was performed in the following way. A 1-ml. aliquot in 5% aqueous sodium bicarbonate, estimated to contain 0.2 to 0.5 mg. of XI, was diluted with 1 ml. of 3 *N* hydrochloric acid, then heated on a steam-bath for 15 minutes to hydrolyze any anil (X) to *p*-aminobenzoyl-L-glutamic acid. This solution was then cooled, diazotized and coupled with N-(1-naphthyl)-ethylenediamine as described by Bratton and Marshall (22). The amount of dye was determined by its extinction at 560 mμ using *p*-aminobenzoyl-L-glutamic acid as a standard.

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Nonaqueous Titrimetric Analysis of Aminophylline

By THOMAS MEDWICK and FREDERICK SCHIESSWOHL

A nonaqueous potentiometric titration is described by which ethylenediamine and theophylline, the components of aminophylline, are titrated differentially as a mixture of bases. Acetic acid is used as the solvent for the ethylenediamine neutralization after which acetic anhydride is added to the system to allow titration of the theophylline, a very weak base. This method was applied to the analysis of aminophylline powder, tablets, ampuls, and suppositories. When compared with the U.S.P. XVI analyses, the nonaqueous approach is simpler since no elaborate sample treatment is needed. The precision of the one titration, nonaqueous procedure is about the same as the U.S.P. XVI analyses which require two titrations to obtain the same data. An alternative approach is suggested for cases, for example, some tablets, where the nonaqueous method is not successful.

VARIOUS APPROACHES to the analysis of aminophylline, a mixture of theophylline and ethylenediamine, have been reported. In the majority, these procedures measure the theophylline which is present and (by appropriate calculation) express the result as aminophylline. Connors (1) discussed the argentometric, ultraviolet spectrophotometric, and other methods which have been used. In the U.S.P. XVI (2–5) the theophylline content of aminophylline in its various forms is determined by an argentometric titration procedure involving several preliminary steps. In aminophylline powder and ampuls only (2, 3) in addition to the theophylline analysis, an ethylenediamine assay is specified.

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Nonaqueous Titrimetric Analysis of Aminophylline

By THOMAS MEDWICK and FREDERICK SCHIESSWOHL

A nonaqueous potentiometric titration is described by which ethylenediamine and theophylline, the components of aminophylline, are titrated differentially as a mixture of bases. Acetic acid is used as the solvent for the ethylenediamine neutralization after which acetic anhydride is added to the system to allow titration of the theophylline, a very weak base. This method was applied to the analysis of aminophylline powder, tablets, ampuls, and suppositories. When compared with the U.S.P. XVI analyses, the nonaqueous approach is simpler since no elaborate sample treatment is needed. The precision of the one titration, nonaqueous procedure is about the same as the U.S.P. XVI analyses which require two titrations to obtain the same data. An alternative approach is suggested for cases, for example, some tablets, where the nonaqueous method is not successful.

VARIOUS APPROACHES to the analysis of aminophylline, a mixture of theophylline and ethylenediamine, have been reported. In the majority, these procedures measure the theophylline which is present and (by appropriate calculation) express the result as aminophylline. Connors (1) discussed the argentometric, ultraviolet spectrophotometric, and other methods which have been used. In the U.S.P. XVI (2–5) the theophylline content of aminophylline in its various forms is determined by an argentometric titration procedure involving several preliminary steps. In aminophylline powder and ampuls only (2, 3) in addition to the theophylline analysis, an ethylenediamine assay is specified.

The xanthines, theophylline, theobromine, and caffeine possess analytically useful acid-base properties. All three xanthines have been found to be very weakly basic, pKb's (aqueous) > 13 (6), and, in addition, theophylline and theobromine are weakly acidic, pKa (aqueous) = 8.6, pKa (aqueous) = 10, respectively. Acetic anhydride has been used as a solvent component in the titration of these compounds as bases. The titrant used exclusively was acetous perchloric acid. Theophylline has been titrated in a 4:1 nitromethane-acetic anhydride solvent (7) and theobromine, dihydroxypropyltheophylline, and caffeine were titrated in mixed solvents containing acetic anhydride and nitromethane, benzene, toluene, or dioxane (8, 9). Similarly, Anastasi, Gallo, and Novacic (10) reported the satisfactory titration of caffeine in an acetic anhydride–acetic acid solvent. Recent work by Ellert, Jasinski, and Pawelczak (11) showed that, although theophylline did not titrate as a base in propionic acid, a propionic acid–propionic anhydride solvent permitted satisfactory titration as a base. Employing the acidic behavior of theophylline, McEniry (12) was able to determine the theophylline content of aminophylline by titrating a sample in dimethylformamide with sodium methoxide titrant.

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is a distinctly basic compound, pKb (aqueous) = 4.07 (13), it should be readily titratable. This communication deals with the selection and analytical use of a nonaqueous solvent system in which ethylenediamine and theophylline, the components of aminophylline, can be determined differentially as bases by a single titration.

EXPERIMENTAL

Pharmaceuticals, Chemicals, and Reagents Used

Aminophylline powder U.S.P., Merck and Co., Inc., Rahway, N. J.; aminophylline tablets, 100 and 200 mg., containing at least 80% anhydrous theophylline; aminophylline injection, 500 mg. in 2 ml., containing at least 80% anhydrous theophylline and benzyl alcohol, 2% v/v; aminophylline suppositories, 0.5 Gm., (cocoa-butter base with paraffin) were employed.

All chemicals were reagent grade. Acetous perchloric acid, *ca.* 0.1 *N*, was prepared according to Fritz (14) and standardized against triphenylguanidine with α-naphtholbenzein indicator. The indicator was a 0.2% w/v solution in glacial acetic acid.

Selection of a Solvent System

The properties of ethylenediamine and theophylline complicate the selection of a suitable titration medium. Although acetic acid (a valuable solvent for the titration of bases) would permit satisfactory determination of the stronger base, ethylenediamine, theophylline is too weakly basic to be determined in this solvent. Theophylline has been successfully determined in an acetic anhydride–containing solvent (7). However, if the anhydride were used here, reaction with ethylenediamine in the sample would destroy the desired basic properties of the amine unless the amine was already titrated or the titration was carried out at a low temperature (15). As a result of these considerations the following approach was developed.

After the aminophylline sample is dissolved in glacial acetic acid, it is potentiometrically titrated with acetous perchloric acid in the presence of an indicator which shows the completion of the ethylenediamine neutralization without the necessity of a preliminary potential (mv.) *versus* titrant added (ml.) plot. A small additional volume of titrant is added, followed by a volume of acetic anhydride. The titration is continued in this new medium until complete. This basic procedure is varied as needed in cases of specific dosage form analysis.

Procedures

The potentiometric titrations were conducted using a Beckman model N pH meter equipped with a glass electrode (Beckman No. 41263) and a sleeve-type calomel electrode (Beckman No. 40463) in which the aqueous saturated potassium chloride solution was replaced with 0.2 *M* acetous lithium chloride. A 10-ml. teflon stopcock Koch microburet was employed.

Aminophylline Powder.—About 0.135 Gm. of aminophylline is accurately weighed and dissolved in 30 ml. of glacial acetic acid in a beaker. After six drops of α-naphtholbenzein indicator solution are added, the solution is potentiometrically titrated with *ca.* 0.1 *N* acetous perchloric acid using the electrode system previously described. When the solution exhibits a green tinge, the volume is recorded and a total of 2 ml. of the titrant is added in two increments. Ten milliliters of acetic anhydride is introduced into the solution; the titration is continued until complete. A plot of the data, mv. observed *versus* ml. of titrant, is constructed and the end points graphically determined (16).

Aminophylline Tablets.—A sample of 20 tablets is accurately weighed, ground to a fine powder, and a sample of the powder equivalent to *ca.* 0.135 Gm. of aminophylline is then accurately weighed into a beaker. Thirty milliliters of glacial acetic acid are added and the analysis is carried out as described above under *Aminophylline Powder* by following the procedure from "After six drops of α-naphtholbenzein . . ."

Aminophylline Injection.—A volume of the injection equivalent to 500 mg. of aminophylline is accurately measured and transferred into a 100-ml. volumetric flask. Acetic acid is used to bring the solution to the mark. A sample of exactly

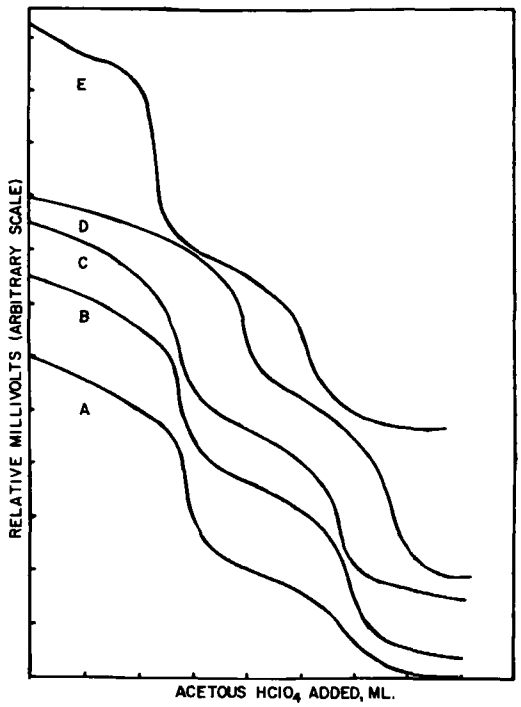

Fig. 1.—Each ordinate division is equivalent to 100 mv. Each abscissa division is equivalent to 2 ml. Curve A–Titration of aminophylline powder in glacial acetic acid. Curve B–Titration of aminophylline powder in glacial acetic acid with the addition of acetic anhydride after the first inflection point. Curve C–Titration of a sample of 200-mg. aminophylline tablets in glacial acetic acid with the addition of acetic anhydride after the first inflection point. Curve D–Titration of a sample of aminophylline injection 500 mg./2 ml. in glacial acetic acid with the addition of acetic anhydride after the first inflection point. Curve E–Titration of a sample of 0.5-Gm. aminophylline suppositories in chloroform-acetic acid with the addition of acetic anhydride after the first inflection point.

25 ml. is transferred to a beaker which contains 5 ml. of glacial acetic acid. The analysis is carried out as described above under *Aminophylline Powder* by following the procedure from "After six drops of α-naphtholbenzein ..." being certain to note the following conditions.

When the injection taken for analysis contains 500 mg. in 2 ml., then 10 ml. of acetic anhydride is used as directed under *Aminophylline Powder*. However, when the injection to be analyzed contains 500 mg. in 20 ml., then 40 ml. of acetic anhydride is added, the remainder of the procedure continuing unchanged.

Aminophylline Suppositories.—A small beaker and glass rod are tared after which five suppositories are placed into the beaker and accurately weighed. The beaker is heated until the suppositories are melted; the melt is allowed to cool without mixing. When solid, an amount of sample equivalent to about 0.5 Gm. of aminophylline is weighed and placed into a beaker. Chloroform, 60 ml., and acetic acid, 40 ml., are added alternately in small quantities and mixed with the sample until solution is completed. The sample solution is transferred quantitatively to a 100-ml. volumetric flask and brought to volume with any chloroform and acetic acid remaining. Chloroform–acetic acid, 3:2 v/v, may be used, if needed, to complete the dilution. A sample of exactly 25 ml. is transferred to a beaker containing 15 ml. of glacial acetic acid. The analysis is carried out as described above under *Aminophylline Powder* by following the procedure from "After six drops of α-naphtholbenzein ..."

U.S.P. Analyses.—Each sample was analyzed by the official U.S.P. XVI methods (2–5).

Calculation for the Nonaqueous Procedures.—

$$\text{Ethylenediamine, \%} = \frac{\text{ml. (first end point)} \times N_{HClO_4} \times 0.03005}{\text{Gm. aminophylline in sample}}$$

$$\text{Theophylline, \%} = \frac{\text{ml. (second end point} - \text{first end point)} \times N_{HClO_4} \times 0.1802}{\text{Gm. aminophylline in sample}}$$

$$\text{Aminophylline dihydrate, \%} = \frac{\text{ml. (second end point)} \times N_{HClO_4} \times 0.1141}{\text{Gm. aminophylline dihydrate in sample}}$$

RESULTS AND DISCUSSION

The titrimetric behavior of aminophylline as a mixture of two bases was observed as expected according to the aqueous pKb values. Although the pKb difference (*ca.* 10 units) is sufficient to permit the bases to be differentiated in glacial acetic acid solvent (Fig. 1, Curve A), it is noted that only the first inflection, representing the neutralization of the stronger base, ethylenediamine, is analytically useful. The second inflection point, assignable to theophylline neutralization, is not satisfactory.

Acetic anhydride has been found to be a valuable solvent for the titration of very weak bases (15, 17). More than simply producing an anhydrous medium by reacting with water (a weak base), this ability has been related to the presence in acetic anhydride of a species more acidic than the solvated proton in acetous perchloric acid (18). Figure 1, curve B, represents the titrimetric behavior of a sample of aminophylline powder in acetic acid solution to which acetic anhydride was added after the first (ethylenediamine) neutralization was completed. It is noted that the character of the second or theophylline neutralization is improved. Both inflections are now analytically suitable.

The nature of Fig. 1, curves B, C, D, and E indicates that satisfactory titrimetric behavior was experienced when aminophylline analyses were carried out on the powder, tablets, injection, and suppositories, respectively. Graphical determination of the end point was possible without difficulty.

The analytical results are summarized in Table I. Each of the samples were analyzed by the official analytical procedures as well as by the nonaqueous titration technique. All of the aminophylline monographs (except for the powder) require the argentometric result (theophylline determination) to be expressed as aminophylline dihydrate. Where possible, the nonaqueous data are presented in the same form as the official analytical results. The nonaqueous calculation is made using the total volume of acid consumed, *i.e.*, the volume needed to neutralize both the ethylenediamine and theophylline. Because of the differential nature of the nonaqueous titration, the percentage of ethylenediamine is calculated from the first end point volume obtained from the same curve.

It is noted that the ethylenediamine content of the

TABLE I.—SUMMARY OF AMINOPHYLLINE ANALYSES[a]

	Nonaqueous			U.S.P. XVI[b]		
	n[d]	Ethylenediamine, %	Xanthine, %	n[d]	Ethylenediamine, %	Xanthine, %
Aminophylline powder U.S.P.	8	12.9 ± 0.2	83.2 ± 1.2 (as theophylline)	3	13.1 ± 0.1 (12.8–14.1)	82.5 ± 0.8 (78 to 83.5) (as theophylline)
Aminophylline tablets, 200 mg.	4	12.6 ± 0.1	98.8 ± 0.1 (as aminophylline dihydrate)	4	...[c]	102.0 ± 0.6 (93–107) (as aminophylline dihydrate)
Aminophylline injection, 500 mg./2 ml.	3	19.4 ± 0.1	81.3 ± 0.4 (as theophylline)	3	19.0 ± 0.1 (18.8–20.1)	102.1 ± 1.3 (93–107) (as aminophylline dihydrate)
Aminophylline suppositories, 500 mg.	4	12.0 ± 0.2	101.7 ± 0.9 (as aminophylline dihydrate)	4	...[c]	102.9 ± 0.2 (90–110) (as aminophylline dihydrate)

[a] Values are means and their standard deviation. [b] Values in parentheses indicate acceptable U.S.P. range. [c] No official analysis specified in this case. [d] Number of determinations is n.

injection is higher than in the other dosage forms when determined by either method. The U.S.P. provides for the addition of excess ethylenediamine to assure a clear solution (3). Thus, when the nonaqueous analysis is carried out, the total volume of acid consumed does not represent aminophylline only but includes the additional ethylenediamine. The calculation reported in Table I, in this instance, is made from the volume of acid used to neutralize the theophylline present and is expressed as percentage of theophylline. The percentage of ethylenediamine is reported also.

When the procedure for the analysis of aminophylline injection is examined, it is noted that the amount of acetic anhydride to be added is variable. Since injections of different concentration are available, the amount of acetic anhydride added must be altered to react with all the water introduced in sampling the aqueous injection solution. In addition to creating a water-free solution, enough excess anhydride remains to be consistent with the 15–20% (by volume) anhydride–acetic acid composition employed in the other dosage form procedures.

Inspection of the data presented in Table I indicate reasonable agreement between the nonaqueous and official methods. The largest deviation exists in the case of the 200-mg. tablets where the nonaqueous value is about 3% lower than the official result. The precision of both methods is found to be about 0.1 to 0.2% for ethylenediamine and about 0.7% for the xanthine (as theophylline or aminophylline dihydrate).

This nonaqueous method was not applicable in the case of some tablets. Although satisfactory results were achieved with 200-mg. tablets, very high results were noted in the analysis of 100-mg. tablets. From the appearance of the titration curves, it was apparent that some tablet constituent other than ethylenediamine and theophylline was acting as a base and contributing to the high results. This behavior was not unexpected and was attributed to the presence of sodium sulfate in the formulation. The interference of various tablet components in acetic acid–nonaqueous titrimetric procedures has been reported (19, 20) and should be anticipated.

Since the nonaqueous method was not successful in the analysis of some tablets, an alternative means of analysis was sought. The official approach to the measurement of ethylenediamine in aminophylline powder and injection employs titration of a sample in water to a methyl orange end point. This simple aqueous titration was found to work well for the determination of ethylenediamine in aminophylline tablets. However, in the present work, better end point detection was achieved by the use of bromcresol green indicator (21). Thus, when the nonaqueous method is unsuccessful, the argentometric or ultraviolet spectrophotometric methods for theophylline determination (1) may be combined with the aqueous titration of ethylenediamine for complete aminophylline analysis.

The nonaqueous procedure offers certain advantages when compared with the official analytical approach. The nonaqueous method is capable of measuring both components of aminophylline in a single titration without elaborate sample treatment. There are fewer reagents needed and the time-consuming heating, cooling, and filtration of the official argentometric assay are eliminated. The simplified procedure is of particular note in the case of the official suppository analysis which involves liquid-liquid extraction as well.

SUMMARY

A time saving, nonaqueous titrimetric procedure has been developed whereby the components of aminophylline, ethylenediamine, and theophylline are titrated differentially as bases during a single titration.

The precision of the described method is similar to the official analysis which requires elaborate sample treatment as well as two titrations to obtain the same data.

The nonaqueous method was not successful in the analysis of certain aminophylline tablets because of the presence of basic tablet components. An alternative approach has been suggested.

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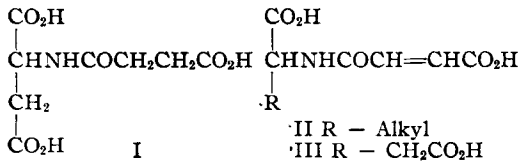
Some Derivatives of Aspartic and Glutamic Acids

By AJAY K. BOSE† and RICHARD E. STRUBE

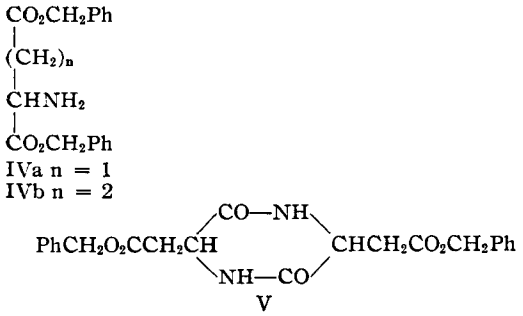
N-Acylation of L-glutamic and L-aspartic acids was carried out under mild conditions *via* their benzyl esters. Anomalous racemization was observed with some aspartic acid derivatives.

In connection with some research on the biogenesis of diaminopimelic acid (1) a sample of N-(β-carboxypropionyl)-L-aspartic acid (I) was required. It has been reported (2) that amino acids in aqueous solution react with maleic anhydride in benzene solution to give the N-acylated derivative II in good yield

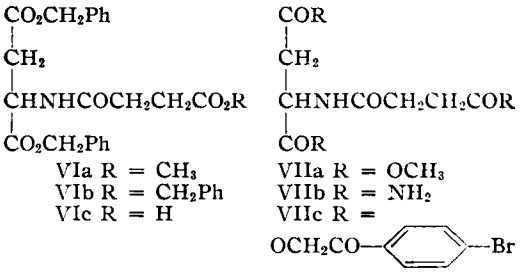


We attempted the preparation of N-(β-carboxyacroyl)-L-aspartic acid (III) by this method with the aim of hydrogenating it to the desired aspartic acid derivative, I; however, neither L-aspartic acid nor its disodium salt reacted with maleic anhydride or succinic anhydride (3). β-Carbomethoxypropionyl chloride and disodium aspartate (4) also failed to lead to the desired product.

In the hope that acylation might be more successful in a homogeneous reaction mixture, it was decided to use dibenzyl L-aspartate (IVa) which is soluble in organic solvents. This ester was obtained as a viscous oil which on long standing deposited a crystalline solid. The diketopiperazine structure V has been assigned to this on the basis of elemental analyses, molecular weight, and infrared spectrum



Dibenzyl L-aspartate (IVa) reacted readily with β-carbomethoxypropionyl chloride and β-carbobenzyloxypropionyl chloride to give the N-acylated products VIa and VIb, respectively. Hydrogenolysis of VIb gave an amorphous product whose infrared spectrum showed the presence of the amide and carboxyl functions expected of I. It was found that IVa condensed with succinic anhydride to give the N-acylated compound VIc in excellent yield. Hydrogenolysis of VIc afforded I as an amorphous material



On treatment of I with diazomethane an oily trimethyl ester (VIIa) was obtained; this gave a crystalline amide (VIIb) by reaction with alcoholic ammonia. Compound I was further characterized by the preparation of a crystalline *p*-bromophenacyl ester (VIIc).

N-Phthaloyl amino acids are of importance in peptide synthesis. Some amino acids, such as L-aspartic and L-glutamic acids, undergo racemization during phthaloylation by the fusion method. Alternative multi-step procedures have been described for the preparation of N-phthaloyl derivatives of L-aspartic and L-glutamic acids (5–10). The availability of dibenzyl L-aspartate made it possible to explore yet another mild method of phthaloylation that has been described recently (11, 12). This method, which involves phthaloylation in toluene solution in presence of triethylamine, was also applied to esters of L-glutamic acid.

EXPERIMENTAL[1]

Benzyl esters of amino acids were prepared as their *p*-toluene-sulfonate salts by using the method of Cipera and Nicholls (13). The free esters, obtained in high yield on shaking the salts with cold sodium bicarbonate solution, were optically active and could

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[1] All melting points are uncorrected.

be used for acylation without further purification. β-Benzyl L-aspartate was prepared by using the procedure described for γ-benzyl L-glutamate (14).

Dibenzyl L-Aspartate *p*-Toluenesulfonate.—*p*-Toluenesulfonic acid monohydrate (60.0 Gm., 0.32 mole), L-aspartic acid (40.0 Gm., 0.30 mole), and benzyl alcohol (100 ml., 104 Gm., 0.96 mole) were placed in a 1-L. flask provided with a mechanical stirrer and a Dean-Stark water separator with condenser. The mixture was heated for 18 hours under vigorous reflux. Fifteen milliliters of water was collected during this period. The clear solution was cooled in ice and the precipitate removed by filtration. The crude product (131 Gm., yield 74%), recrystallized twice from water containing 5% of ethanol, yielded 82 Gm. (41%); m.p. 157–158°, $[\alpha]_D^{25}$ + 1° (95% EtOH).

Anal.—Calcd. for $C_{25}H_{27}NO_7S$: C, 61.85; H, 5.61; S, 6.59. Found: C, 61.44; H, 5.69; S, 6.61.

The preparation of dibenzyl DL-aspartate *p*-toluenesulfonate by a similar procedure has been described (15).

Dibenzyl L-Aspartate (IVa).—One hundred grams (0.206 mole) of dibenzyl L-aspartate *p*-toluenesulfonate was added to a cold mixture of 500 ml. of ether and 100 ml. of water in a separator and shaken. A cold, nearly saturated solution of 45.0 Gm. (0.363 mole) of sodium carbonate monohydrate was then added and the mixture shaken vigorously for a few minutes. The ether layer was separated and the aqueous layer was extracted several times with ether. The combined ether extracts were dried over anhydrous sodium sulfate. On removing the solvent under reduced pressure at room temperature, 57.4 Gm. (88%) of a slightly yellow oil (IVa) was obtained. Purification of this product by distillation under reduced pressure could not be accomplished because the compound decomposed at about 90° with the formation of succinic anhydride, m.p. 119°.

For identification, 3.0 Gm. of the oil IVa was converted to its hydrochloride (2.6 Gm., 60%) which was recrystallized from methanol-ether, m.p. 129.5 to 130° [lit. (16) m.p., 124–125°], $[\alpha]_D^{25}$ + 1 (95% ethyl alcohol).

Anal.—Calcd. for $C_{18}H_{20}ClNO_4$: C, 61.79; H, 5.76; Cl, 10.14; N, 4.01. Found: C, 61.70; H, 6.02; Cl, 9.93; N, 4.03.

After standing for several weeks at room temperature, the compound IVa deposited crystals which were removed by filtration. Compound V melted at 157–158° after washing with acetone and recrystallization from ethanol.

Anal.—Calcd. for $C_{22}H_{22}N_2O_6$: C, 64.38; H, 5.40; N, 6.83; mol. wt., 410. Found: C, 64.32; H, 5.57; N, 6.95; mol. wt., 434.

$\nu_{max.}^{Nujol}$ in cm.$^{-1}$: 3290, 3150 (N—H); 1735 (SH), 1730 (ester CO); 1688, 1655 (amide CO).

Hydrogenolysis of Dibenzyl L-Aspartate.—The ester IVa (3.13 Gm.) was dissolved in 25 ml. of absolute methanol and hydrogenated at an initial pressure of 20 lbs./sq. in. in the presence of 1.5 Gm. of 10% palladium-on-charcoal. The catalyst and the precipitate were removed by filtration and then extracted with 500 ml. of hot water. The clear aqueous solution was lyophilized. The rotation and infrared spectrum of the product were identical with that of L-aspartic acid.

β-Benzyl L-Aspartate.—Fifty milliliters of concentrated sulfuric acid followed by 500 ml. of benzyl alcohol were added carefully to 500 ml. of ether. The ether was removed under reduced pressure and 66.5 Gm. (0.50 mole) of L-aspartic acid was added. The solution was kept at room temperature for 20 hours, then cooled to 0°, and a mixture of 250 ml. of pyridine in 1000 ml. of ethanol (96%) was added under vigorous stirring. The precipitate (71 Gm., 63%) was removed by filtration and washed with ether after standing overnight in the refrigerator. Recrystallization from 1000 ml. of water containing 10 ml. of pyridine gave 37 Gm. (33%) of pure XI: m.p. 211.5 to 212.5° dec.; $[\alpha]_D^{25}$ + 27° (1 *N* HCl).

Anal.—Calcd. for $C_{11}H_{13}NO_4$: C, 59.18; H, 5.87; N, 6.28. Found: C, 58.86; H, 6.01; N, 6.33.

Benzyl Esters of L-Glutamic Acid.—Under conditions similar to those described above for L-aspartic acid, the reaction of 33.0 Gm. (0.224 mole) of L-glutamic acid, 75 ml. (0.723 mole) of benzyl alcohol, and 45.0 Gm. (0.238 mole) of *p*-toluenesulfonic acid monohydrate in benzene for 13 hours afforded 98 Gm. (88%) of dibenzyl L-glutamate *p*-toluenesulfonate, m.p. 122.5 to 128.5°. On recrystallization from hot water, the m.p. was raised to 139.5 to 141.5°.

Anal.—Calcd. for $C_{26}H_{29}NO_7S$: C, 62.71; H, 5.87; N, 2.80; S, 6.41. Found: C, 62.47; H, 5.76; N, 3.05; S, 6.66.

When 14.7 Gm. (0.100 mole) of L-glutamic acid, 30 ml. of benzyl alcohol, 19.2 Gm. (0.110 mole) of *p*-toluenesulfonic acid monohydrate, and 75 ml. of benzene were allowed to react for only 4 hours, the product amounted to 43.5 Gm. of a white solid. On recrystallization from ethanol-ether, 33 Gm. of a solid, m.p. 147–153°, was obtained. A solution of 2.00 Gm. of this material in 20 ml. of 95% alcohol was stirred; 5 ml. of pyridine was added dropwise. After storage overnight in an icebox, the solid was filtered, washed with cold alcohol, and dried to give 0.68 Gm. (43%) of γ-benzyl L-glutamate (IXc), m.p. 162–164°, $[\alpha]_D^{25}$ + 19° (c = 2.62 in acetic acid). A sample of this ester prepared by following the method of Guttmann and Boissonnas (14) showed m.p. 168° and $[\alpha]_D^{25}$ + 18.6° (c = 5.855 in acetic acid).

Using the same method as for preparing IVa, 54.0 Gm. of dibenzyl L-glutamate *p*-toluenesulfonate was converted into 34.4 Gm. (97%) of the free diester IVb. This ester was characterized by its conversion in a nearly quantitative yield to its hydrochloride, m.p. 99–100°, $[\alpha]_D^{25}$ + 9° (0.1 *N* HCl) [lit. (17) m.p., 100–102°, $[\alpha]_D^{25}$ + 9.4°].

Anal.—Calcd. for $C_{19}H_{22}ClNO_4$: C, 62.71; H, 6.09; Cl, 9.74. Found: C, 62.77; H, 6.25; Cl, 9.67.

L-Leucine Benzyl Ester *p*-Toluenesulfonate.—This compound was prepared from L-leucine (26.2 Gm., 0.200 mole), benzyl alcohol (35 Gm., 0.32 mole), and *p*-toluene sulfonic acid monohydrate (40.0 Gm., 0.230 mole) by the esterification procedure described above. The yield of the crude product was 50 Gm. (62%). After recrystallization from water, the m.p. was 211.5 to 212.5° dec.

Anal.—Calcd. for $C_{20}H_{27}NO_5S$: C, 61.04; H, 6.92; N, 3.56; S, 8.15. Found: C, 61.21; H, 7.05; N, 3.84; S, 8.22.

N-(β-Carbomethoxyprepionyl)-L-aspartic Acid,

Dibenzyl Ester (VIa).—A solution of 1.9 Gm. (0.015 mole) of sodium carbonate monohydrate in 4.5 ml. of water was added to a solution of 9.4 Gm. of freshly prepared IVa in 50 ml. of ether. A solution of 3-carbomethoxypropionyl chloride (18) in 50 ml. of ether was added dropwise to this mixture in the course of 30 minutes at 15–17°. After stirring for another 30 minutes the ether layer was separated, dried over anhydrous sodium sulfate, and the solvent removed under reduced pressure. The residual oil (11.7 Gm., 91%) solidified after standing for 2 days under reduced pressure over concentrated sulfuric acid. Recrystallization from petroleum ether gave 9.1 Gm. (71%) of VIa, m.p. 57–58°.

Anal.—Calcd. for $C_{23}H_{25}NO_7$: C, 64.62; H, 5.89; N, 3.28. Found: C, 64.73; H, 5.99; N, 3.19.

β-Carbobenzyloxypropionyl Chloride.—A mixture of benzyl hydrogen succinate (19) (6.24 Gm., 0.040 mole) and thionyl chloride (9.5 Gm., 0.080 mole) was heated in a bath at 35–40° for 100 minutes. The excess thionyl chloride was removed under reduced pressure at room temperature. The colorless oil could not be purified by distillation under reduced pressure and was, therefore, used directly for the preparation of VIb.

N-(β-Carbobenzyloxypropionyl)-L-aspartic Acid, Dibenzyl Ester (VIb).—A solution of 2.0 Gm. (0.016 mole) of sodium carbonate monohydrate in 4.5 ml. of water was added to a solution of 9.4 Gm. of freshly prepared IVa in 50 ml. of methylene chloride. A methylene chloride solution of 3-carbobenzoxypropionyl chloride (prepared freshly from 6.24 Gm. (0.040 mole) of the corresponding acid) was added dropwise to this mixture in the course of 30 minutes at 10–15°. Proceeding in a similar manner as described above for compound VIa, 13.6 Gm. (90%) of VIb, m.p. 70 to 71.5°, was obtained.

Anal.—Calcd. for $C_{29}H_{29}NO_7$: C, 69.17; H, 5.81; N, 2.78. Found: C, 69.08; H, 5.49; N, 2.74.

N-(β-Carboxypropionyl)-L-aspartic Acid, Dibenzyl Ester (VIc).—A solution of 28.8 Gm. of freshly prepared IVa and 9.0 Gm. (0.090 mole) of succinic anhydride in 300 ml. chloroform was heated under reflux for 30 minutes. After removing the solvent by distillation, an oil (36.6 Gm.) was obtained which crystallized on scratching with a glass rod and adding some petroleum ether. Recrystallization from carbon tetrachloride gave 34.9 Gm. (96%) of a white solid, m.p. 97–98°.

Anal.—Calcd. for $C_{22}H_{23}NO_7$: C, 63.91; H, 5.61; N, 3.39. Found: C, 63.76; H, 5.63; N, 3.36.

N-(β-Carboxypropionyl)-L-aspartic Acid (I).—(*a*) Compound VIb (0.5 Gm., 0.001 mole), dissolved in 150 ml. of absolute methanol, was catalytically hydrogenated in the presence of 2.0 Gm. of 10% palladium-on-charcoal and at an initial pressure of 50 lbs./sq. in. The hydrogenation was complete in 5–10 minutes. The catalyst was removed by filtration and the solvent was distilled under reduced pressure over phosphorus pentoxide. Attempts to crystallize the oil were unsuccessful even after several months of storage.

Anal.—Calcd. for $C_8H_{11}NO_7$: C, 41.20; H, 4.76; N, 6.01. Found: C, 41.70; H, 4.91; N, 5.67.

(*b*) Compound VIa was hydrogenated as described above and the product was isolated as an amorphous solid.

Anal.—Calcd. for $C_8H_{11}NO_7$: C, 41.20; H, 4.76; N, 6.01. Found: C, 41.40; H, 5.12; N, 5.53.

N-(β-Carboxypropionyl)-L-aspartic Acid, Trimethyl Ester (VIIa).—Compound VIc (1.0 Gm.) was hydrogenated as described above; to the methanol solution of the product (*ca.* 3 ml.) was added dropwise with stirring at 0–5° a cold solution of diazomethane in ether until a yellow color remained. After standing for 10 minutes, dilute 1:4 hydrochloric acid was added until the yellow color of the excess diazomethane had disappeared. The ether layer was washed with water and dried over anhydrous sodium sulfate. After removing the solvent by distillation a colorless oil was obtained, the infrared spectrum of which indicated that no free carboxyl group was present. The product was used directly for preparing compound VIIb.

N-(β-Carboxypropionyl)-L-aspartic Acid, Triamide (VIIb).—Compound VIIa was dissolved in 3 ml. of absolute ethanol and 10 ml. of ethanol saturated with ammonia was added. The flask was stoppered and left at room temperature for 1 week. The precipitate formed was removed by filtration, washed with absolute ethanol, and recrystallized from water. The yield was 0.47 Gm. (82% based on compound VIc), m.p. 220.5 to 221.0° dec.

Anal.—Calcd. for $C_8H_{14}N_4O_4$: C, 41.73; H, 6.13. Found: C, 41.55; H, 6.05.

N-(β-Carboxypropionyl)-L-aspartic Acid, Tri-(*p*-bromophenacyl) Ester (VIIc).—Compound VIb (1.0 Gm.) was hydrogenated as mentioned before; after removing the solvent, the acid was neutralized with 0.1 *N* sodium hydroxide and then lyophilized. Water (10 ml.) and a solution of 2.98 Gm. (0.01 mole) of *p*-bromophenacylbromide in 100 ml. of absolute ethanol were added. The solution was heated on the steam bath for 3 hours, filtered, and cooled in the refrigerator for several hours. The crystalline product formed was recrystallized from absolute ethanol, m.p. 138–139° dec.

Anal.—Calcd. for $C_{32}H_{26}Br_3NO_{10}$: C, 46.62; H, 3.68; Br, 29.08; N, 1.69. Found: C, 46.58; H, 3.50; Br, 29.40; N, 1.78.

N-Phthaloyl-L-aspartic Acid.—(*a*) A mixture of 4.85 Gm. of dibenzyl L-aspartate (IVa), 2.3 Gm. (0.015 mole) of phthalic anhydride, 0.5 ml. of triethylamine, and 100 ml. of toluene was heated under reflux for 3.5 hours in a flask fitted with a Dean-Stark water separator. On working up the reaction product as described in (*b*), 6.3 Gm. of an oil was obtained which on hydrogenation gave 3.7 Gm. of a solid. Crystallization from hot water following decolorization with Darco charcoal gave a solid, m.p. 223–226°, $[\alpha]_D^{25} - 39°$ (c = 0.639 in ethanol) [lit. (10) m.p., 197°, $[\alpha]_D^{25} - 58°$ (methanol)]. The infrared spectrum of this material was identical with that of phthaloyl aspartic acid.

(*b*) Finely powdered phthalic anhydride (1.48 Gm., 0.0100 mole) was dissolved with shaking in a mixture of 250 ml. of toluene and 10 ml. of triethylamine. A 4.85-Gm. (0.0100 mole) quantity of dibenzyl L-aspartate *p*-toluenesulfonate, was added to this solution; the mixture was heated under reflux for 2.5 hours in a flask fitted with a Dean-Stark water separator. Within a few minutes of heating all suspended solid dissolved. At the end

of the reaction a small second layer of liquid below the toluene layer dissolved in water.

The upper toluene layer was washed, dried, and evaporated to give 3.9 Gm. of a viscous, yellow oil which had a satisfactory infrared spectrum [$\nu_{max.}^{Nujol}$ in cm.$^{-1}$ 1780, 1730 (phthalimido carbonyls); 1710 (ester carbonyl)]. On hydrogenation of this oil in methanol solution in the presence of 0.8 Gm. of 10% palladium-on-charcoal catalyst, nearly two moles of hydrogen were absorbed. After filtration the reaction mixture was evaporated and 2.5 Gm. of a white solid was obtained. After one crystallization from hot water, the m.p. of this product was 221–223° (softens at 180°) and $[\alpha]_D^{25}$ − 40°.

After two more recrystallizations from hot water the melting point changed to 228 to 229.5° and $[\alpha]_D^{25}$ became −9° (c = 0.627 in 95% ethyl alcohol).

Evidently DL-phthaloylaspartic acid is less soluble in water than L-phthaloylaspartic acid and recrystallization from water has resulted in the enrichment of the proportion of the DL-acid. This is in keeping with the findings of Schilling and Strong (20) concerning the comparative solubility of DL- and L-phthaloylglutamic acids.

Anal.—Calcd. for $C_{12}H_9NO_6$: C, 54.76; H, 3.45; N, 5.32. Found: C, 54.81; H, 3.71; N, 5.19.

In a second experiment starting with 10 Gm. of dibenzyl L-aspartate *p*-toluenesulfonate, 3.7 Gm. (67%) of phthaloylaspartic acid, m.p. 223–225°, $[\alpha]_D^{25} \backsim 0°$, was obtained.

(*c*) A mixture of 3.87 Gm. of dibenzyl L-aspartate (IVa), 1.75 Gm. (0.0118 mole) of phthalic anhydride, and 50 ml. of N,N-dimethylformamide was heated to boiling for 45 minutes and then poured on cracked ice. This mixture was extracted with methylene chloride. On evaporating the methylene chloride, a dark material was extracted with ether. The ether solution was thoroughly washed with water, dried, and evaporated to give 4.4 Gm. of a yellow viscous oil which was hydrogenated. The product (2.65 Gm.), m.p. 223–225°, was optically inactive.

(*d*) A mixture of 4.63 Gm. of dibenzyl L-aspartate (IVa), 3.00 Gm. (0.0203 mole) of phthalic anhydride, and 30 ml. of acetic acid was heated under reflux for 8 hours and then poured into 250 ml. of water. The mixture was extracted with methylene chloride and the extract washed with dilute sodium bicarbonate solution and then with water, dried, and evaporated to give 5.5 Gm. of a colorless, viscous oil. On hydrogenation, 3.0 Gm. of a solid, m.p. 225–227°, $[\alpha]_D^{25} \sim 0°$, was obtained.

Diethyl N-Phthaloyl-L-aspartate.—A suspension of 11.3 Gm. (0.050 mole) of diethyl L-aspartate hydrochloride and 7.5 Gm. (0.051 mole) of phthalic anhydride in 150 ml. of toluene and 10 ml. of triethylamine was refluxed for 2.5 hours in a flask fitted with a water separator. Nearly the calculated amount of water separated. The reaction mixture was filtered and the solid so obtained was washed with benzene and dried to give 6.5 Gm. (95%) of triethylamine hydrochloride. The filtrate was washed with dilute hydrochloric acid and with water, dried over anhydrous magnesium sulfate, and evaporated to give 14.0 Gm. (92.5%) of a slightly yellow, heavy oil. On distillation a colorless oil was obtained (nearly quantitative recovery) which had a satisfactory infrared spectrum and $[\alpha]_D^{25}$ −45° (c = 0.7 in ethanol, [lit. (10), $[\alpha]_D^{?}$ −40° (ethanol)].

N-Phthaloyl-L-glutamic Acid.—(*a*) Using the general procedure described above, L-glutamic acid *γ*-benzyl ester (IIc) was converted to an oily phthaloylation product which on hydrogenation gave a colorless oil. This oil was dissolved in hot water; on seeding, a white solid (68.5% overall yield), m.p. 156–159°, was obtained. On evaporation the mother liquor gave an oil that refused to crystallize. The solid after recrystallization from hot water had a m.p. 158–160° and $[\alpha]_D^{25}$ − 45° (c = 0.773 in ethanol) [lit. (8), m.p. 160–161°; $[\alpha]_D$ − 43° (95% ethyl alcohol)].

Anal.—Calcd. for $C_{13}H_{11}NO_6$: C, 56.32; H, 4.00; N, 5.05. Found: C, 56.56; H, 4.18; N, 4.85.

$\nu_{max.}$ in cm.$^{-1}$: 3200–2500 (bonded hydroxy), 1771 (CO), and 1742 (SH) (carbonyl).

(*b*) To the clear solution obtained on shaking 1.48 Gm. (0.0100 mole) of phthalic anhydride with 150 ml. of toluene and 5 ml. of triethylamine, 5.13 Gm. (0.0103 mole) of dibenzyl L-glutamate *p*-toluenesulfonate was added. After heating for a few minutes all solid disappeared and a two-phase liquid mixture was obtained; this was heated under reflux for 3 hours in a flask fitted with a Dean-Stark water separator. After cooling, the reaction mixture was successively washed with water, dilute hydrochloric acid and water, dried, and evaporated. A 3.5 Gm. quantity of a colorless viscous oil with the infrared spectrum expected for N-phthaloyl-glutamic acid dibenzyl ester (VIIId) was obtained. This oil was hydrogenated in methanol solution in the presence of 0.5 Gm. of 10% palladium-on-charcoal catalyst and the product was purified by crystallization from hot water. The yield of N-phthaloyl L-glutamic acid (VIIIc), m.p. 158–160°, $[\alpha]_D^{25}$ − 44° (c = 0.833 in ethanol), was 1.4 Gm. (50%). The infrared spectrum of the product was satisfactory.

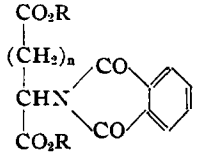
N-Phthaloyl-L-leucine.—Using the one-step phthaloylation method described above, L-leucine benzyl ester *p*-toluenesulfonate was converted to the oily N-phthaloyl-L-leucine benzyl ester which was used without purification for hydrogenation. After crystallization from aqueous methanol, the product, m.p. 119–121°, $[\alpha]_D^{25}$ − 24° (c = 0.5 in ethanol) [lit. (21) m.p., 118.5 to 119.5°, $[\alpha]_D^{26.5}$ − 24°], was obtained in 38% overall yield.

DISCUSSION

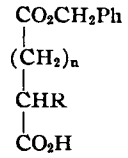
Sachs and Brand (22) have described a procedure for the preparation of dibenzyl L-glutamate hydro chloride. In our hands this method led only to the hydrochloride of L-glutamic acid. The *p*-toluenesulfonate of dibenzyl L-glutamate, which is easily prepared in good yield, can be converted into the hydrochloride or directly used in place of the hydrochloride.

The reaction of dibenzyl L-glutamate *p*-toluenesulfonate (IVb) [or *γ*-benzyl L-glutamate (IXc)] with an acid anhydride followed by hydrogenolysis appears to be a convenient synthesis of optically active pure N-acyl L-glutamic acid. It is noteworthy, however, that the same sequence of reactions with dibenzyl L-aspartate *p*-toluenesulfonate led to a racemized product. When the *β*-benzyl ester of L-aspartic acid was employed as the starting material, the extent of racemization was much reduced. It is interesting to note that when diethyl L-aspartate

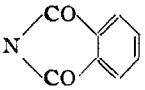
hydrochloride was substituted for the dibenzyl ester, the N-phthaloylaspartic acid diethyl ester (VIIIc) was formed without any racemization



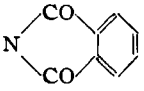
VIIIa n = 1, R = CH_2Ph
VIIIb n = 1, R = H
VIIIc n = 1, R = C_2H_5
VIIId n = 2, R = CH_2Ph
VIIIe n = 2, R = H



IXa n = 1, R = NH_2

IXb n = 1, R = 

IXc n = 2, R = NH_2

IXd n = 2, R = 

To examine the possibility that benzyl esters of amino acids may be prone to racemization, the *p*-toluenesulfonate of L-leucine benzyl ester was phthaloylated using the procedure of Bose, Greer, and Price (11) and the product hydrogenated. The N-phthaloyl-L-leucine so obtained was optically pure.

In the light of the results with benzyl esters of L-leucine and L-glutamic acid, the behavior of the benzyl esters of L-aspartic acid during phthaloylation is anomalous. In particular, it is difficult to see why β-benzyl L-aspartate should racemize to a limited extent during the phthaloylation and hydrogenation sequence.

After the completion of this work the preparation of N-phthaloyl L-glutamic acid (23) and N-phthaloyl L-aspartic acid (24) has been reported using a new method (25).

SUMMARY

L-Aspartic acid failed to condense with succinic or maleic anhydride, but the desired N-(β-carboxypropionyl)-aspartic acid could be prepared by the reaction of succinic anhydride with dibenzyl L-aspartate, followed by hydrogenolysis of the ester group.

The preparation of mono and dibenzyl esters of L-aspartic and L-glutamic acids and the benzyl esters of L-leucine is described. N-Phthaloyl derivatives of glutamic acid and aspartic acid were prepared under mild conditions *via* their benzyl esters. Optically pure N-phthaloyl L-glutamic acid was obtained this way, but anomalous racemization was observed with some aspartic acid derivatives.

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Rates of Hydrolysis of Carbamate and Carbonate Esters in Alkaline Solution

By LEWIS W. DITTERT† and TAKERU HIGUCHI

The rate of the alkaline hydrolysis of several aliphatic and aromatic carbamate and and carbonate esters was studied at various temperatures. The reactions were found to be first order with respect to both hydroxyl ion and the ester. The data seemed to indicate two mechanisms for the hydrolysis of the carbamates: that initiated by an hydroxyl ion attachment on the central carbon atom followed by a carbamate ion intermediate, and that involving direct ionization of an amide hydrogen followed by formation of an isocyanate intermediate. Since the latter mechanism appears to proceed with much greater ease for carbamates of strongly electrophilic alcohols, the N-unsubstituted and N-monosubstituted carbamates of these compounds undergo cleavage at rates several orders of magnitude greater than those of the corresponding disubstituted carbamates.

ALTHOUGH several drugs are marketed which contain the carbamate ester linkage, *e.g.*, physostigmine, neostigmine, carbachol, etc., no exhaustive kinetic or mechanistic study on the general stability of this linkage toward alkaline hydrolysis appears to have been carried out. The specific stability of physostigmine in solution has, however, been the object of many qualitative investigations (1–6). Ellis and his co-workers (7) in a quantitative study showed that the destruction of physostigmine in aqueous solution was first order in hydroxyl ions and first order in physostigmine. A physostigmine analog, neostigmine methylsulfate (8), was reported by Morch (9) to be hydrolyzed in very dilute alkaline solutions under autoclave conditions, but Chaiken (10) was unable to find any decomposition of an analogous compound (a dimethylcarbamate) in pH 7.4 phosphate buffer at 38°. The results of these studies indicate that the dimethylcarbamate esters are much more stable toward alkaline hydrolysis than are the N-methylcarbamate esters.

More recently, because of their potent anticholinesterase activity, carbamates have been employed as insecticides, *e.g.*, Sevin,[1] Isolan,[2] Pyrolan,[2] Pyramat,[2] etc. Casida and his coworkers (11) have studied the alkaline hydrolysis of these and similar compounds and have also found the dimethylcarbamate esters to be more stable than the N-methylcarbamate esters.

It has been known for many years that carbamate esters in alkaline solution hydrolyze according to

$$R{-}O{-}\overset{\displaystyle O}{\overset{\|}{C}}{-}NR'_2 + 2OH^- = ROH + CO_3^= + HNR'_2$$

Although Werner (12), in 1918, proposed a mechanism for the destruction of urethane in aqueous alkali at 100°, no exhaustive kinetic or mechanistic study on urethane appears to have been carried out since that time.

It was the purpose of the present investigation to explore the general kinetics and mechanisms of the alkaline hydrolysis of urethane, ethyl N-methylcarbamate, ethyl dimethylcarbamate, phenyl carbamate, phenyl N-methylcarbamate, phenyl dimethylcarbamate, *p*-nitrophenyl carbamate, *p*-nitrophenyl N-methylcarbamate, *p*-nitrophenyl dimethylcarbamate, phenyl ethyl carbonate, and *p*-nitrophenyl ethyl carbonate, and to determine the effect of temperature and structure on the hydrolytic reaction.

EXPERIMENTAL

Materials

Ethyl N-Methylcarbamate and Ethyl Dimethylcarbamate.—The compounds were synthesized by the method of Schreiner (13) and purified by fractional distillation through a 30-cm. Vigreaux column. The following fractions were collected: ethyl N-methylcarbamate 167–168° (lit. 170° (13)), ethyl dimethylcarbamate 142–144° (lit. 139–140 (13)).

Phenyl N-Methylcarbamate.—The compound was synthesized by the method of Schreiner (13) and purified by repeated crystallization from 5% benzene in petroleum ether, m.p. 85–87°.

Phenyl Dimethylcarbamate and p-Nitrophenyl Dimethylcarbamate.—The compounds were synthesized by reacting dimethyl carbamyl chloride with the appropriate phenol in pyridine solution. Phenyl dimethylcarbamate was recrystallized repeatedly from petroleum ether, m.p. 44 to 45.5° (lit. 44–45° (14)). *p*-Nitrophenyl dimethylcarbamate was recrystallized repeatedly from ethanol, m.p. 107–109°.

Phenyl Ethyl Carbonate and p-Nitrophenyl Ethyl Carbonate.—The compounds were prepared

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TABLE I.—PSEUDO FIRST-ORDER RATE CONSTANTS AND SECOND-ORDER RATE CONSTANTS FOR THE DISAPPEARANCE OF URETHANE ($k_{URETHANE}$) AND THE APPEARANCE OF CARBONATE IONS ($k_{CARBONATE}$) IN STRONGLY BASIC SOLUTIONS OF URETHANE AT 30°C.

N OH^-	$k_{urethane} \cdot [OH^-]$ ($Hr.^{-1}$)	$k_{urethane}$ ($L. \cdot Mole^{-1} \cdot Hr.^{-1}$)	$k_{carbonate} \cdot [OH^-]$ ($Hr.^{-1}$)	Approx. Lag Time, $Hr.^{-1}$
0.10	1.2×10^{-2}	0.12	1.4×10^{-2}	2
0.15	1.8×10^{-2}	0.12	...	...
0.20	2.4×10^{-2}	0.12	2.4×10^{-2}	5
0.25	3.1×10^{-2}	0.12	...	...
0.30	3.8×10^{-2}	0.13	3.8×10^{-2}	6
0.35	4.4×10^{-2}	0.13	...	...
0.40	5.1×10^{-2}	0.13	5.2×10^{-2}	8
0.50	...	...	5.3×10^{-2}	11
0.70	...	...	5.0×10^{-2}	14
1.00	...	...	3.4×10^{-2}	17
		av. 0.12		

by reacting ethyl chloroformate with the appropriate phenol in pyridine solution. Phenyl ethyl carbonate was purified by fractional distillation through a 30-cm. Vigreaux column, b.p. 226–228° (lit. 227–230° (15)). *p*-Nitrophenyl ethyl carbonate was recrystallized repeatedly from ethanol, m.p. 64–66° (lit. 67–68° (16)).

p-Nitrophenyl Carbamate and p-Nitrophenyl N-Methylcarbamate.—The compounds were prepared by nitration of the corresponding phenyl esters after the method of Ransom (16). *p*-Nitrophenyl carbamate was recrystallized repeatedly from ethanol, m.p. 110–140° (lit. 161° (17)). *p*-Nitrophenyl N-methylcarbamate was recrystallized repeatedly from ether-acetone solution, m.p. 105–109°.

Procedures

1. The Rate of Disappearance of Urethane from Strongly Basic Solutions at 30°.—The reaction mixture was prepared by dissolving urethane in sodium hydroxide–barium hydroxide solution of the desired normality. Periodically, 5 to 20-ml. portions of this solution containing about 15 mg. of urethane were neutralized by dropwise addition of 60% perchloric acid and distributed in 1 Gm. of silicic acid per ml. of urethane solution. The silicic acid was slurried with chloroform, quantitatively transferred to a chromatography column, packed down, and developed with 300 to 500 ml. of chloroform. The eluate was boiled down to a small volume, and 10 ml. of 0.1 *N* perchloric acid in glacial acetic acid and 25 ml. of chlorobenzene were added. The mixture was boiled until the temperature of the solution reached 104°. The solution was refluxed for 2 hours at 104°, cooled, and titrated with 0.05 *N* sodium acetate in glacial acetic acid to the α-naphthol benzein end point. The amount of perchloric acid consumed represented the amount of urethane present in the aliquot.

2. Rate of Appearance of Carbonate Ions in Strongly Basic Solutions of Urethane at 30°.—The reaction mixture was prepared as described in the preceding paragraph, and 20-ml. aliquots of the solution were filled into 8-dr. vials with a hypodermic syringe. The vials were sealed with rubber stoppers and placed in a 30° thermostat. Periodically, the vials were removed from the thermostat; sufficient barium chloride solution was added to precipitate all the carbonate ion which had formed. The contents of the vials were then filtered through Pyrex glass medium porosity fritted disk Buchner funnels. The barium carbonate precipitate was washed well and dissolved in standard hydrochloric acid. This solution was washed through the funnel and the excess acid titrated with standard carbonate-free sodium hydroxide solution to the bromcresol green end point.

3. Rate of Alkaline Hydrolysis of Urethane, Ethyl N-Methylcarbamate, and Ethyl Dimethylcarbamate in Strongly Basic Solutions at Various Temperatures.—The reaction mixture was prepared by dissolving the ester in sodium hydroxide–barium hydroxide solution so that the concentration of hydroxyl ions was twice the concentration of the ester. Five-milliliter aliquots of this solution were filled into 5-ml. ampuls with a hypodermic syringe, and the ampuls were sealed and placed in a thermostat at the desired temperature. At zero time, and periodically thereafter, the ampuls were removed from the bath and crushed under standard hydrochloric acid. The excess acid was titrated with standard carbonate-free sodium hydroxide solution to the bromcresol green end point.

4. Rate of Alkaline Hydrolysis of Various Aromatic Carbamates and Carbonates at Various Temperatures in Buffered and Strongly Basic Solutions.—The reactions were followed spectrally by measuring

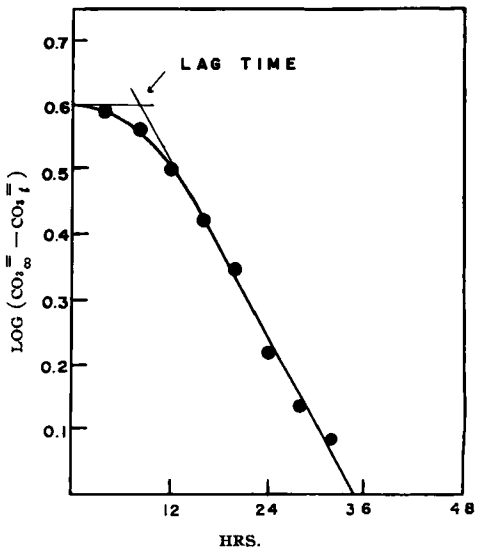


Fig.1.—Plot showing the rate of appearance of carbonate ions in a 0.4 *N* OH^- solution of urethane at 30°C.

TABLE II.—SECOND-ORDER RATE CONSTANTS FOR THE ALKALINE HYDROLYSIS OF ALIPHATIC CARBAMATE ESTERS IN STRONGLY BASIC SOLUTION

Ester	T, °C.	k(L.·Mole^{-1}·Min.$^{-1}$)	Apparent ΔHa (Kcal./Mole)
Urethane	55°	2.4×10^{-2}	
	30°	2.0×10^{-3}	20
	25°[a]	1.2×10^{-3}	
Ethyl	55°	5.1×10^{-3}	
N-methyl-	30°	5.5×10^{-4}	17.5
carbamate	25°[a]	3.4×10^{-4}	
Ethyl	96°	4.2×10^{-2}	
dimethyl-	55°	3.0×10^{-3}	
carbamate	25°[a]	2.7×10^{-4}	15.5

[a] The k's at 25°C. were extrapolated from the higher temperature data.

the absorbance due to the phenate ion (at 235 mμ wavelength) or the *p*-nitrophenate ion (at 400 mμ wavelength) using a model 11 MS Cary recording spectrophotometer. The reactions were allowed to proceed until no further change in the absorbance could be detected. Pseudo first-order rate constants were determined from the slopes of plots of log ($A_\infty - A_t$) *versus* time. Borate and phosphate buffers and sodium hydroxide–barium hydroxide solutions were employed and several pH's or hydroxyl ion concentrations were studied for each compound. All buffer results were extrapolated to infinite dilution.

RESULTS AND DISCUSSION

Experimental studies on the hydrolytic rate of urethane and various substituted carbamates strongly suggest that, as would be expected, the initial cleavage occurred at the CO—O bond.

Urethane.—The pseudo first-order rate constants ($k_{\text{urethane}} \cdot [OH^-]$) for the rate of disappearance of urethane from strongly basic solutions at 30° and the dependency of this reaction on the base concentration are shown in Table I. These data were procured by *Procedure 1*. A plot of pseudo first-order rate constant *versus* hydroxyl ion concentration yielded a straight line through the origin with a slope of unity, showing that the reaction was first order with respect to hydroxyl ions. The second-order rate constant (k_{urethane}) for this reaction at 30° is also shown in Table I. This rate constant corresponds to the rate of the initial attack on the urethane molecule.

A typical plot showing the rate of appearance of carbonate ions in strongly basic solutions of urethane at 30° is shown in Fig. 1. These data were procured by *Procedure 2*. The reactions were allowed to proceed until no further change in the amount of barium carbonate could be detected. The plot of log

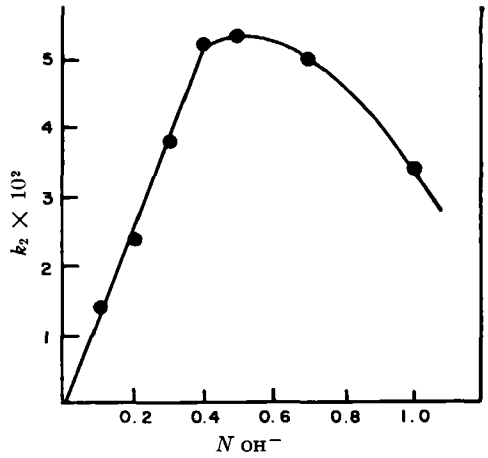


Fig. 2.—Plot showing the relationship between pseudo first-order rate constants for the rate of appearance of carbonate ions in strongly basic solutions of urethane at 30°C. and hydroxyl ion concentration.

($CO_{3\infty}^{=} - CO_{3t}^{=}$) *versus* time, shown in Fig. 1, goes through a lag period, then a straight line portion. The dependency of the slopes of the straight line portions, calculated as pseudo first-order rate constants, on the base concentration is shown in Table I and Fig. 2. The dependency of the lag time on the base concentration is also shown in Table I.

Faurholt (18) pointed out that in solutions of pH above 12.5, the rate of conversion of carbamate ions into carbonate ions and ammonia decreases as the pH increases. This is shown in Table I as an increase in the lag time with increasing hydroxyl ion concentration. The effect is also shown in Fig. 2 as a decrease in $k_{\text{carbonate}}$ with increasing hydroxyl ion concentration above 0.5 N OH$^-$. The fact that k_{urethane} equals $k_{\text{carbonate}}$ between 0.1 and 0.4 N OH$^-$ shows that the rate of rupture of the CO—O bond in the ester linkage is the rate-determining step in the appearance of carbonate ions at these OH$^-$ concentrations. The decrease in $k_{\text{carbonate}}$ with increasing hydroxyl ion concentration beyond 0.5 N OH$^-$ indicates that at these OH$^-$ concentrations the conversion of carbamate ions into carbonate ions is the rate-determining step in the appearance of carbonate ions.

Werner (12) stated that in refluxing sodium hydroxide solution the major intermediate in the hydrolysis of urethane was alkali cyanate. When a solution of urethane in 0.5 N OH$^-$, which had stood at room temperature until the precipitate of barium carbonate was in evidence, was tested for cyanate ions by Werner's method (19), the test was so faint that it could hardly be distinguished from the blank. According to Jensen (20), the decomposition of cyanate ion in such strongly basic solutions is extreme-

TABLE III.—SECOND-ORDER RATE CONSTANTS FOR THE ALKALINE HYDROLYSIS OF AROMATIC CARBAMATE ESTERS

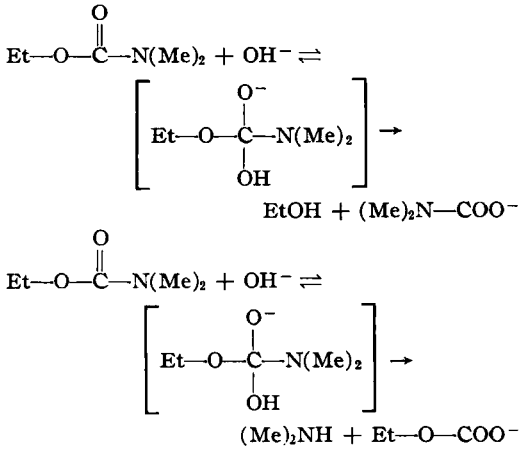
Ester	k(L.·Mole^{-1}·Min.$^{-1}$) 34°C.	25°C.	Apparent ΔHa (Kcal./Mole)
p-Nitrophenyl dimethylcarbamate	5.5×10^{-2}	2.4×10^{-2}	17
p-Nitrophenyl N-methylcarbamate	3.9×10^{4}	3.5×10^{4}	2
p-Nitrophenyl carbamate	1.5×10^{7}	1.5×10^{7}	2
Phenyl dimethylcarbamate	6.0×10^{-3}	2.8×10^{-3}	16
Phenyl N-methylcarbamate	1.9×10^{2}	1.2×10^{2}	8
Phenyl carbamate	2.2×10^{4}	1.6×10^{4}	7

ly slow. If cyanate ion had been the true intermediate in the hydrolysis of urethane at 30°, carbonate ions probably never would have been detected.

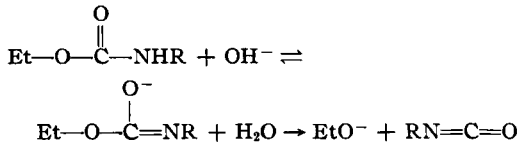
Effect of Temperature on the Rate of Alkaline Hydrolysis of Urethane, Ethyl N-Methylcarbamate, and Ethyl Dimethylcarbamate in Strongly Basic Solutions.—The second-order rate constants for the alkaline hydrolysis of the three aliphatic carbamate esters at several temperatures are shown in Table II. These data were procured by *Procedure 3*.

This method for following the aliphatic carbamates was considered completely reliable for studies at any hydroxyl ion concentration, even though appreciable quantities of carbamate ions may have been present in the reaction mixture. The aliquots to be analyzed were first placed in strong acid solution which, according to Faurholt (18), would instantaneously decompose any carbamate ions into ammonia and carbon dioxide and include them in the titer. According to Jensen (20), any cyanate ion present would also have been rapidly converted into ammonia and carbon dioxide under these conditions, although the change would not have been instantaneous. The decomposition of urethane itself was studied by *Procedure 3* at 30°, and the resulting k was exactly the same as the value previously determined by following the disappearance of the ester.

Methylation of the amide nitrogen of urethane produced significant changes in the hydrolytic rate, but the changes were not sufficient to indicate major changes in the basic mechanism. In the case of ethyl dimethylcarbamate, the only mechanism possible is a direct hydroxyl ion attack on the carbonyl carbon atom followed by either a dimethylcarbamate ion or a monoethylcarbonate ion intermediate



Faurholt (18) has shown that the dimethylcarbamate ion does not exist in solution under any conditions and (21) that the monoethylcarbonate ion is rapidly converted into carbamate ions and ethyl alcohol under the conditions of these experiments. Thus, the initial hydroxyl ion attack on the ethyl dimethylcarbamate ester is probably followed by complete disintegration of the molecule. In the urethane and ethyl N-methylcarbamate cases the formation of an isocyanate intermediate is possible



but the rate constants for these compounds were so close to that for ethyl dimethylcarbamate that this possibility seems remote. The apparent heats of activation for the three compounds are essentially equal, suggesting no major difference in mechanism between them. The small differences in rates which were observed are probably because of the inductive and steric effect of the methyl groups.

Rate of Alkaline Hydrolysis of Various Aromatic Carbamates.—The second-order rate constants obtained for several aromatic carbamates are shown in Table III. These data were procured by following the appearance of the phenate or p-nitrophenate ions spectrally and therefore correspond to the rate of cleavage of only the CO—O bond.

Unlike the case of urethane, methylation of the amide nitrogen of phenyl carbamate and p-nitrophenylcarbamate has a very marked effect on the hydrolytic rate. The second-order k for phenyl carbamate is nearly 10^7 times that for phenyl dimethylcarbamate. The second-order k for p-nitrophenyl carbamate is nearly 10^9 times that for p-nitrophenyl dimethylcarbamate. The apparent heats of activation for the hydrolysis of these two types of carbamates are also very different. These facts strongly suggest some shift in the mechanism of the hydrolysis. It has already been pointed out in the case of ethyl dimethylcarbamate, that the hydrolysis of the dimethylcarbamates can only occur by direct hydroxyl ion attack on the carbonyl carbon, probably followed by complete disintegration of the molecule. It therefore seems likely that the alternative mechanism, *i.e.*, the formation of an isocyanate intermediate, may be responsible for the great increase in the hydrolytic rate seen with the aromatic N-unsubstituted and N-monosubstituted carbamate esters. If the hydrolysis proceeds according to

TABLE IV.—SECOND-ORDER RATE CONSTANTS FOR THE ALKALINE HYDROLYSIS OF CARBONATE ESTERS

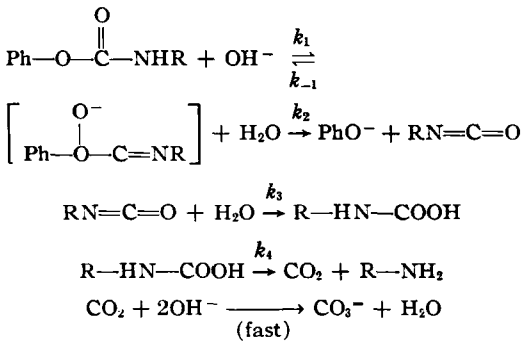
Ester	k(L.·Mole^{-1}·Min.$^{-1}$) 34°C.	25°C.	Apparent ΔHa (Kcal./Mole)
p-Nitrophenyl ethyl carbonate	400	320	5
Phenyl ethyl carbonate	37	24	9
Diethyl carbonate[a]	5.5(35°C.)	2.8	13

[a] After Miller and Case (22).

TABLE V.—INFLUENCE OF THE ELECTROPHILICITY OF THE ALCOHOLIC OR PHENOLIC GROUPS ON THE STABILITY OF ETHYL CARBONATE AND ACETATE ESTERS TOWARD HYDROXYL ION ATTACK

Ester	pKa of ROH	k(L.·Mole^{-1}·Min.$^{-1}$) (25°C.)
p-Nitrophenyl ethyl carbonate	~6	320
Phenyl ethyl carbonate	~10	24
Diethyl carbonate[a]	~16	2.8
p-Nitrophenyl acetate[b]	~6	1600 (22°C.)
Phenyl acetate	~10	...
Ethyl acetate[c]	~16	5.6 (21°C.)

[a] Miller and Case (22). [b] Casida, *et al.* (11). [c] Warder (23).



it is possible that the initial ionization of the carbamate ester has a negative temperature coefficient, since the apparent heat of activation of the overall hydrolysis reaction is rather small. The relative closeness of the rates and apparent heats of activation for phenyl carbamate and phenyl N-methylcarbamate and for *p*-nitrophenyl carbamate and *p*-nitrophenyl N-methylcarbamate suggests that the isocyanate mechanism is dominant in these cases.

Rate of Alkaline Hydrolysis of Various Aromatic Carbonates.—The pseudo first-order rate constants and second-order rate constants obtained for two aromatic ethyl carbonates are shown in Table IV. These data were procured by following the appearance of the phenate or *p*-nitrophenate ions spectrally and therefore correspond to the rate of rupture of only the CO—O bond vicinal to the phenol group.

Miller and Case (22) studied the rate of alkaline hydrolysis of diethyl carbonate and determined a value of 2.8 L. · mole^{-1} · minute^{-1} for the second-order rate constant for the initial cleavage at 25° and an apparent heat of activation of 13 Kcal./mole for this reaction. The differences in rates of hydrolysis of the various carbonate esters seem to parallel the electrophilicity of the alcoholic or phenolic moieties. This

TABLE VI.—SECOND-ORDER RATE CONSTANTS FOR THE ALKALINE HYDROLYSIS OF CARBAMATE AND CARBONATE ESTERS IN 0.05 *M* PHOSPHATE BUFFER AT pH 7.8 AND 22°C. AFTER CASIDA, *et al.* (11)

Ester	k(L. · Mole^{-1} · Min.$^{-1}$)
p-Nitrophenyl dimethylcarbamate	2.0×10^{-2}
p-Nitrophenyl N-methylcarbamate	1.3×10^{5}
Phenyl dimethylcarbamate	3.9×10^{-3}
p-Nitrophenyl ethyl carbonate	5.5×10^{2}

TABLE VII.—INFLUENCE OF THE ELECTROPHILICITY OF THE ALCOHOLIC OR PHENOLIC GROUPS ON THE STABILITY OF DIMETHYLCARBAMATE AND ETHYL CARBONATE ESTERS TOWARD HYDROXYL ION ATTACK

Ester	pKa of ROH	k(L. · Mole^{-1} · Min.$^{-1}$) (25°C.)
p-Nitrophenyl dimethylcarbamate	~6	2.4×10^{-2}
Phenyl dimethylcarbamate	~10	2.8×10^{-3}
Ethyl dimethylcarbamate	~16	2.7×10^{-4}
p-Nitrophenyl ethyl carbonate	~6	320
Phenyl ethyl carbonate	~10	24
Diethyl carbonate	~16	2.8

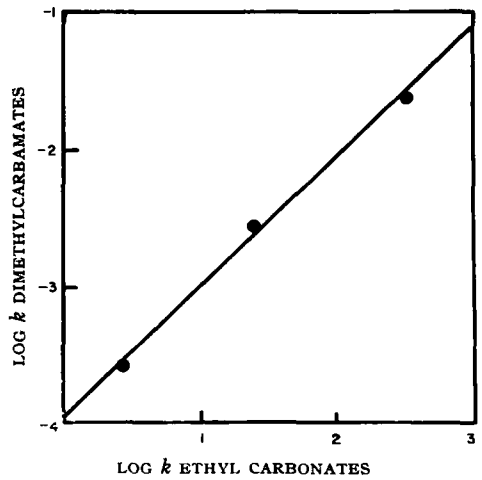
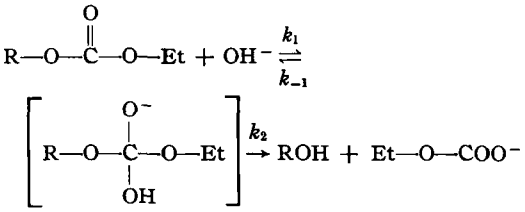


Fig. 3.—Plot showing the proportionality of effect of R—O— groups on the rate of hydrolysis of dimethylcarbamate and ethyl carbonate esters.

effect is illustrated in Table V. It can be seen that the effect of the alcoholic or phenolic groups on the stability of the ethyl carbonate esters and the acetate esters toward hydroxyl ion attack is essentially the same. This suggests that the increased hydrolytic rate in each case is because of an increased polarization of the carbonyl carbon atom which facilitates the initial hydroxyl ion attack. In the carbonate case, this initial attack is followed by a monoalkylcarbonate ion intermediate, probably monoethylcarbonate ion

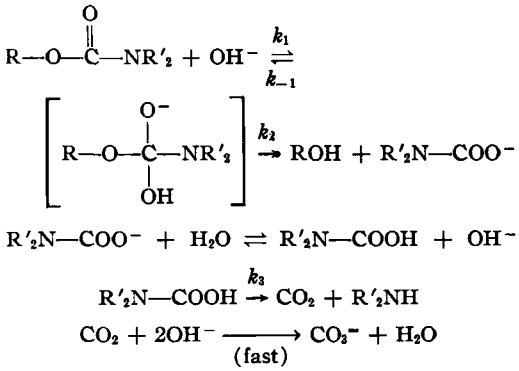


and the effect of the R group suggests that the rate-determining step in this mechanism is probably k_1. Faurholt (21) has already shown that the resulting monoethylcarbonate ion is rapidly converted to carbonate ions and ethyl alcohol in the solutions employed in our studies. Thus, the carbonate esters can be considered to be completely destroyed after the initial cleavage.

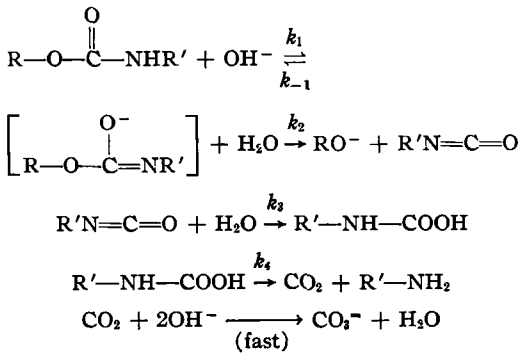
Comparison with the Work of Casida, et al.—Casida and his co-workers (11) have studied the hydrolysis of some of the compounds listed in Tables III and IV in 0.05 *M* phosphate buffer at pH 7.8 and 22°. Their results are shown in Table VI. The rate constants in Table VI are somewhat higher than might be expected from the data of Tables III and IV. This is probably because of buffer catalytic effects.

CONCLUSIONS

On the basis of the data presented above, two mechanisms appear to be responsible for the alkaline hydrolysis of the carbamate esters: (*a*) hydroxyl ion attack followed by a carbamate ion intermediate



(b) hydroxyl ion attack followed by an isocyanate intermediate



The second mechanism appears to predominate in the extremely fast reactions of the N-unsubstituted and N-monosubstituted aromatic carbamates, whereas the first mechanism appears to be the only one possible in the slower reactions of the dimethylcarbamates. The data seem to indicate that mechanism (a) predominates in the hydrolysis of all the aliphatic carbamates. The apparent heats of activation for the compounds which proceed *via* mechanism (a) are of the same order of magnitude as those which might be expected for ordinary hydrolytic reactions. The extremely low apparent heats of activation for the compounds which proceed *via* mechanism (b) seem to indicate that some step in this mechanism has a negative temperature coefficient. That step is very likely the initial ionization of the carbamate ester, since in this step a molecule of water is formed releasing 13 Kcal. of energy.

The electrophilicity of the alcoholic or phenolic moiety appears to exert considerable influence on the stability of the dimethylcarbamate and ethyl carbonate esters toward hydroxyl ion attack. This effect is illustrated in Table VII and Fig. 3 and is probably the result of an increased polarization of the carbonyl carbon atom, since more electrophilic R—O— groups cause greater polarization of the carbonyl group. The Hammett-type plot of log k for a dimethylcarbamate ester *versus* log k for the corresponding ethyl carbonate ester, shown in Fig. 3, is a straight line with a slope very nearly unity, showing that the effect of the alcoholic or phenolic group follows a linear free-energy relationship (24). Since the polarity of the carbonyl group seems largely to determine the rate, k_1 is probably the rate-determining step in mechanism (a).

TABLE VIII.—INFLUENCE OF THE ELECTROPHILICITY OF THE ALCOHOLIC OR PHENOLIC GROUPS ON THE STABILITY OF CARBAMATE AND N-METHYLCARBAMATE ESTERS TOWARD HYDROXYL ION ATTACK

Ester	pKa of ROH	k(L. · Mole^{-1} · Min.$^{-1}$) (25°C.)
p-Nitrophenyl N-methylcarbamate	~6	3.5×10^{4}
Phenyl N-methylcarbamate	~10	1.2×10^{2}
Ethyl N-methylcarbamate	~16	3.4×10^{-4}
p-Nitrophenyl carbamate	~6	1.5×10^{7}
Phenyl carbamate	~10	1.6×10^{4}
Ethyl carbamate (urethane)	~16	1.2×10^{-3}

The effect of the R—O— group is even more marked in the case of intermediate isocyanate formation, *i.e.*, mechanism (b). This is illustrated in Table VIII. The greater effect in this case is probably because this mechanism requires the loss of the R—O$^-$ ion, and *p*-nitrophenate ion is a better leaving group than phenate ion. This suggests that k_2 is probably the rate-determining step in mechanism (b). The two aliphatic compounds are included in Table VIII to show that they do not fit this theory. This is further evidence that the N-unsubstituted and N-monosubstituted aliphatic compounds probably hydrolyze *via* the carbamate ion intermediate, *i.e.*, mechanism (a), at ordinary temperatures. It should be noted that the k's for the aliphatic compounds at 25° are practically equal. The small differences are probably because of the inductive and steric effects of the methyl groups. Even the apparent heats of activation for all the aliphatic compounds are essentially the same and correspond most closely to those values determined for the compounds which can only hydrolyze *via* mechanism (a).

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Chelatometric Determination of Ferrous Iron with 2-Pyridinealdoxime as an Indicator

By SANFORD BOLTON

Ferrous iron may be quantitatively determined by titration into a known EDTA solution containing 2-pyridinealdoxime as an indicator. The principal advantages of this method over oxidation-reduction procedures are that the reagents are more stable and reducing substances, in general, do not interfere.

CLASSIC METHODS for the quantitative determination of ferrous iron have usually involved oxidation-reduction titrations. In these procedures, reducing substances tend to interfere, and the titrating solutions are often unstable, requiring frequent standardizations. A spectrophotometric approach, utilizing the 2-pyridine ketoxime-ferrous chelate, has been proposed by Banerjea and Tripathi (1); however, this type of assay has the disadvantages of being cumbersome and/or requiring special equipment. Because of the advantages of convenience, rapidity, and specificity claimed for chelatometric titrations, much recent work has been reported on this approach to the analysis of metallic ions (2). Notably lacking in the literature, however, is a simple titrimetric chelatometric procedure for determining ferrous iron. The present report describes a titrimetric assay for ferrous iron with 2-pyridinealdoxime (2-PA) as an indicator.

Solutions of ferrous iron form a deep red-colored chelate with 2-PA (3) which is easily detected at low concentrations. A solution of unknown concentration of iron is titrated into a solution of known concentration of EDTA containing 2-PA. The iron first combines stoichiometrically with the EDTA, and the subsequent formation of the red color of the Fe^{++}-2-PA chelate denotes the end point.

This assay method was applied to several official and commercial preparations containing ferrous salts and the results compared favorably with the official assays and/or label-stated quantities.

METHOD

Reagents.—2-Pyridinealdoxime, m.p. 111–113°; indicator solution consisting of a 0.1% solution of 2-PA; EDTA, analytical reagent grade were employed. The known solution of EDTA was prepared by dissolving 4.380 Gm. of EDTA with the aid of three equivalents of NaOH per equivalent of EDTA in enough distilled water to make 500 ml., to yield a solution of approximately 0.03 *M* (disodium EDTA also may be used). This solution was standardized against a known ferrous solution prepared from analytical grade iron wire; 0.50 *M*, pH 5 acetate buffer; salicylic acid, citric acid, and ascorbic acid, all U.S.P. grade; iron wire, analytical reagent grade. All other salts were analytical reagent grade.

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TABLE I.—ASSAY OF FERROUS AMMONIUM SULFATE SOLUTIONS

Assay with Ceric Nitrate, Molar Ferrous	Assay with EDTA and 2-PA, Molar Ferrous	Recovery, %
0.0150	0.0151	100.7
0.00750	0.0076	101.3
0.00375	0.0037	99
0.0600	0.5975	99.6

TABLE II.—TEMPERATURE EFFECTS ON THE ASSAY OF FERROUS AMMONIUM SULFATE SOLUTIONS

Approximate Temp., °C.	Ferrous Theoretical, *M*	Ferrous Observed, *M*	Recovery, %
25	0.0300	0.0310	103.3
35	0.0300	0.0306	102
50	0.0300	0.0301	100.3
65	0.0300	0.0300	100
70	0.0300	0.0300	100
85	0.0300	0.0297	99

Procedure.—An appropriate sample of the ferrous salt or the ferrous containing dosage form was dissolved in distilled water, filtered if necessary, and made to volume in a volumetric flask. For best results the final concentration of iron was found to be approximately 0.03 *M*. This solution was the titrant. Five milliliters of pH 5 acetate buffer were added to a mixture of 1 ml. of 2-PA indicator solution and 2–4 ml. of the known EDTA solution. This mixture was heated to approximately 50–70° and titrated immediately with the iron solution. The solution turned from a pale yellow-green to pink at the end point. The reaction was stoichiometric; the iron consumed was equivalent to the EDTA

$$\text{ml.}_{\text{EDTA}} \times M_{\text{EDTA}} = \text{ml.}_{\text{iron}} \times M_{\text{iron}}$$

The results of this method were compared to results obtained by titration with ceric nitrate and are shown in Table I. These and subsequently reported values are an average of at least three determinations.

Temperature Effect.—The results of the effect of temperature on the appearance of the end point are shown in Table II. Below 50° the end point appeared prematurely, probably because the Fe^{++}-2-PA chelate once formed is not easily dissociated

TABLE III.—VOLUME EFFECTS ON THE ASSAY OF FERROUS ION

Approx. Total Soln. Vol. at End Pt.	Indicator Vol.	Ferrous Theoretical, *M*	Ferrous Observed, *M*
12	2	0.0150	0.0153
12	1/2	0.150	0.0148
12	1	0.0300	0.0301
17	1	0.0300	0.0299
22	1	0.0300	0.0294
22	2	0.0300	0.0298
32	2	0.0300	0.0296
32	1	0.0300	gradual end pt.

at the pH of the assay. It was extremely difficult to eliminate the red color of the chelate, even in the presence of EDTA. The application of heat apparently hastened the equilibrium between the iron and the chelating agents.

pH Effect.—At a pH of 4.5 or below, the end point was gradual and indistinct. At pH's greater than 6, the avidity of the ferrous ion for 2-PA was great enough to produce its characteristic color before the theoretical end point. A satisfactory end point was produced in the range of approximately pH 4.5 to 5.5.

Volume Effect.—Known amounts of distilled water were added to the titration vessel to determine the effect of increased volume on the end point. The results shown in Table III indicate that the end point was slightly delayed at large solution volumes. This observation suggests that the pink color produced at the end point cannot be immediately detected in dilute solutions. In titrations where the total volume of solution at the end point was 22 ml. or more, the addition of an extra milliliter of indicator yielded good results. The effect of indicator volume on the titration was similar to the dilution effect (see Table III). Too little indicator delayed detection of the end point, while too much favored production of the Fe^{++}-2-PA complex with a resultant premature end point.

TABLE IV.—ASSAY OF FERROUS IN THE PRESENCE OF FERRIC ION

Ferric Determined by EDTA-Salicylic Acid Titration, *M*	Total Iron by EDTA-2PA Titration, *M*	Ferrous, *M* (Col. 3 less Col. 2)	Ferrous Theoretical, *M*	Ferric Theoretical, *M*
0.0210	0.0312	0.0102	0.0100	0.0207
0.0105	0.03065	0.02015	0.0200	0.0103
0.0056	0.03055	0.0250	0.0250	0.0052
0.0022	0.0306	0.0284	0.0281	0.0019

INTERFERING SUBSTANCES

Metal Ion Interference.—Metal ions which have high chelate stability constants with EDTA interfered with the assay by preferentially binding the EDTA. The following ions interfered: Mn^{++}, Zn^{++}, Cd^{++}, Ni^{++}, Cu^{++}, Co^{++}, and Fe^{3+} (see below). In the presence of 0.03 *M* Fe^{++}, Ca^{++} interfered above 0.05 *M*. Two molar KNO_3, 3 *M* NaCl, and 1 *M* $MgSO_4$ did not interfere.

Calcium Interference.—Since calcium salts are often ingredients in iron-containing dosage forms, an effort was made to analyze ferrous ion in the presence of calcium. An excess of Na_2SO_4 was added to a known Fe^{++}-Ca^{++} solution; the solution was filtered and made up to volume. Analysis showed that this procedure yielded accurate results for 0.03 *M* Fe^{++} solutions in the presence of at least 0.80 *M* Ca^{++}.

Ferric Interference.—Although Fe^{3+} interfered, the assay gave accurate results of total iron present up to at least a 3:2 ratio of Fe^{3+} to Fe^{++}. A chelatometric procedure based on that suggested by Cheng, *et al.* (4), in combination with the present assay, could be used to determine Fe^{++} in the presence of Fe^{3+}. Two to four milliliters of an approxi-

TABLE V.—ASSAY OF DOSAGE FORMS

Dosage Form	Total Iron, 2-PA-EDTA Tit.	Ferric, Sal. acid-EDTA Tit.	Other Assay Method
Ferrous sulfate syrup U.S.P.	4.12 Gm./100 ml.	negligible	N.F., 4.08 Gm./100 ml.
Ferrous iodide syrup U.S.P.	67 mg./ml.	negligible	N.F., 67.0 mg./ml.
Ferrous gluconate capsules, 325 mg.	332 mg.	13 mg.	U.S.P., 338 mg.
Ferrous lactate drops, 25 mg./ml.	26.9 mg./ml.	uncertain	Ceric, no good
Iron-amino acid complex, 40 mg./ml.	40.9 mg./ml.	uncertain	Ceric[a], 42.4 mg./ml.
Vitamin-iron capsules[b] Ferrous sulfate, 160–200 mg.	167 mg.	uncertain	...
Vitamin-iron tablets$_1$[b] Ferrous sulfate, 300 mg.	310.3 mg.	uncertain	...
Tablets$_2$[b], Ferrous lactate, 30 mg.	31.6 mg.	20 mg.[c]	...
Prepared mixture[b] Ferrous sulfate, 300 mg.	297.6 mg.	uncertain	...

[a] This high value is probably because of unknown additives in the preparation.
[b] Composition of vitamin products:

	Capsules	Tablets$_1$	Tablets$_2$	Prepared Mixture
Desicated liver	325 mg.	100 mg	...	350 mg.
B_{12}	1.7 mcg.	1/3 U.S.P. u.	...	1 mcg.
B_1	2 mg.	2 mg.	...	10 mg.
B_2	2 mg.	2 mg.	...	10 mg.
B_6	1 mg.	...	...	5 mg.
Niacin	10 mg.	10 mg.	...	50 mg.
Folic acid	0.4 mg.	2 mg.	...	3 mg.
C	50 mg.	50 mg.	...	150 mg.
Pantothenic acid	2 mg.	...	...	25 mg.
Calcium lactate	...	...	350 mg.	...
A	...	...	1500 U.S.P. u.	...
D	...	...	150 U.S.P. u.	...
$Al(OH)_3$	...	...	150 mg.	...
Ethyl amino benzoate	...	...	35 mg.	...

[c] These tablets were at least 5 years old.

mately 0.03 *M* iron solution in 0.01 *N* HCl is titrated with EDTA using salicylic acid as an indicator. The disappearance of the red-violet (Fe^{3+}-salicylic acid chelate) color denotes the end point. At this low pH, Fe^{++} combines to a very slight degree with EDTA and the assay gives only Fe^{3+} concentration. The concentration of total iron present and of Fe^{3+} can be used to determine ferrous ion by difference. If the unknown solution has a color of its own, this method fails because the end point in the Fe^{3+} titration cannot be accurately observed. Results of this method are shown in Table IV.

Chelating Agent Interference.—Two chelating agents often found in iron preparations, which might interfere with the assay by binding ferrous ion, are citric acid and ascorbic acid. In known samples containing 0.03 *M* Fe^{++}, citric acid interfered when present in concentrations greater than 0.03 *M*. Ascorbic acid did not affect the assay in concentrations up to at least 0.50 *M*.

ASSAY OF COMMERCIAL PREPARATIONS

Several U.S.P. and commercially available products were assayed for iron content to test the applicability of the method to more complex preparations. Liquid dosage forms were appropriately diluted so that the final concentration of iron was approximately 0.03 *M*. Solid dose forms were prepared by filtering an aqueous suspension of pulverized tablets or capsule contents through a sintered-glass funnel and making up to volume in a volumetric flask. If calcium was present, sodium sulfate was added prior to filtration, as previously described.

Since no other assay method could be conveniently used, the analysis of commercial preparations by this method could only be compared to the labeled amount of iron. Therefore, a mixture was prepared from vitamin capsules and folic acid tablets to contain a known quantity of ferrous sulfate; the assay showed good recovery for ferrous ion (see Table V).

Whenever possible, known assay methods were used to verify the results of the chelatometric titration. Although the commercial liquid iron preparations tested contained only a single active ingredient, titration with ceric nitrate failed, perhaps because of interfering additives. Also the presence of ascorbic acid in other preparations precluded the use of ceric nitrate.

Although some of the diluted iron solutions were colored, the end point of the 2-PA titration was readily distinguishable. However, ferric ion could not be determined in these same preparations because the end point with salicylic acid at pH 2 is not sharp enough to be observed when interfering colors are present.

Results of these assays are shown in Table V.

SUMMARY

When compared to oxidation-reduction and other official methods for the determination of ferrous ion the titrimetric chelatometric assay described in this report presents a more advantageous method under certain conditions: (*a*) Reducing substances, which interfere in oxidation-reduction methods, usually become innocuous in the chelatometric titration. These include such substances as ascorbic acid and possibly certain fillers and flavors in dosage forms. In the case of ferrous iodide syrup N.F., this assay gave accurate results, which compared favorably to the N.F. method and which obviated much of the tedium associated with the official assay. (*b*) The solutions used in the chelatometric titration are extremely stable. In addition, EDTA can be purchased in a virtually 100% pure state; standardized EDTA solutions corresponded very closely to the amount of EDTA used to prepare these solutions. (*c*) Total iron content may be determined even in the presence of large amounts of ferric ion.

Oxidation-reduction methods are more efficacious when ferrous is to be determined in the presence of ferric ion. Also, certain chelating metals and chelating agents interfere with the chelatometric titration. In these cases, oxidation-reduction assay methods can be used to advantage.

Under certain conditions, the procedure for ferrous determination described in this report is advantageous compared to established methods and presents an approach which may be utilized where other methods fail.

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Preparation of Tritio-Cholecalciferol

(Tritiated Vitamin D_3)

By C. T. PENG

Cholecalciferol was tritiated by exposure to tritium gas at −198° (liquid nitrogen). Pure tritiated vitamin D_3 with a specific activity of 216 μc./mg. was obtained following equilibration with solvents and purification by successive recrystallization from acetone at −80° and column chromatography on Florisil until a constant specific activity was achieved. The formation of radiation decomposition products was minimal because of using low temperature during tritiation. A possible explanation for this low temperature effect is given.

BIOLOGICALLY important compounds have frequently been randomly labeled with tritium by the Wilzbach gas exposure method (1). This method, however, has not been widely used with unsaturated compounds because tritium atoms add across the —C=C— bond giving rise to saturated products of extremely high specific activity (2) which are difficult to remove.

The addition reaction of tritium was first observed by Jones, *et al.* (2), with methyl oleate which, on tritiation, yielded methyl tritio-stearate as the only radioactive product. However, in other unsaturated compounds such as cholesterol, isobutylene (3), 1-hexene, 1,5-hexadiene (4), methyl linolenate (5), etc., substitution as well as addition of tritium occurred during the labeling process. While the mechanism of tritiation by Wilzbach method has been studied in homogeneous systems of methane-tritium (6), ethylene-tritium (7), and propane-tritium (8) and found to involve decay- and β-labeling, the mechanism of tritiation in heterogeneous systems remains obscure (9). Moreover, no systematic investigation has been made of the mechanism of hydrogenation of the —C=C— bond by exposure to tritium gas and the effect of changing the reaction parameters, *viz.*, tritium pressure, temperature coefficient, etc.

In this laboratory, tritio-cholecalciferol was prepared by exposure to tritium gas at −198°. This low temperature, as discussed later, minimizes molecular decomposition by radiation because of an enhancement of the "cage" effect and reduces the formation of radioactive impurities. Purification of the tritiated vitamin D_3 was effected by extraction, column chromatography, and recrystallization.

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MATERIALS

All solvents used were reagent grade and were redistilled. Peroxide in ethyl ether was removed by passage through a column of basic alumina. Silica gel G, prepared for thin-layer chromatography according to Stahl (10), was obtained from E. Merck Co. Florisil, activated at 260°, was purchased from Floridin Co., Tallahassee, Fla.

EXPERIMENTAL

All manipulations involving tritiated vitamin D were carried out in an atmosphere of nitrogen and in low actinic glassware, or in glassware protected from light by wrapping in aluminum foil to minimize oxidation and photodecomposition.

Tritiation.—Approximately 500 mg. of vitamin D_3 (Mann Research Laboratories, New York, N. Y.) was exposed to 50 curies of tritium gas at a pressure of 130 mm. Hg at −198° (liquid nitrogen) for 39 days. The vitamin sample was introduced into the tritiation chamber shown in Fig. 1 through the open end *A* which was subsequently sealed. The sample-filled chamber was then attached at *T* to the pumping arm of a Toepler pump in a high vacuum line and evacuated to a pressure of 10^{-6} mm. Hg (11). Tritium gas, obtained from the Oak Ridge National Laboratory, was admitted without further purification into the tritiation chamber. After filling with the gas, the chamber was detached from the vacuum line by sealing off the capillary constriction at *D*.

The sealed tritiation chamber containing vitamin D_3 and tritium gas was immersed in liquid nitrogen (−198°) in a dewar flask and kept there for the duration of the exposure time.

To terminate the labeling process, the tritium gas, together with any radioactive gaseous products formed by radiation decomposition during exposure, was removed by breaking seal *B* of the tritiation chamber in the high vacuum line system.

Purification.—Purification of the tritiated vitamin D_3 consists of removing labile tritium activity and then eliminating radioactive impurities. Complete recovery at each step of purification was not attempted because it was more important that trace impurities of high specific activity be completely separated from the parent compound rather than high yields be obtained.

The unpurified tritiated vitamin D_3 was repeatedly dissolved in dry acetone and evaporated to dryness to remove labile tritium activities. The

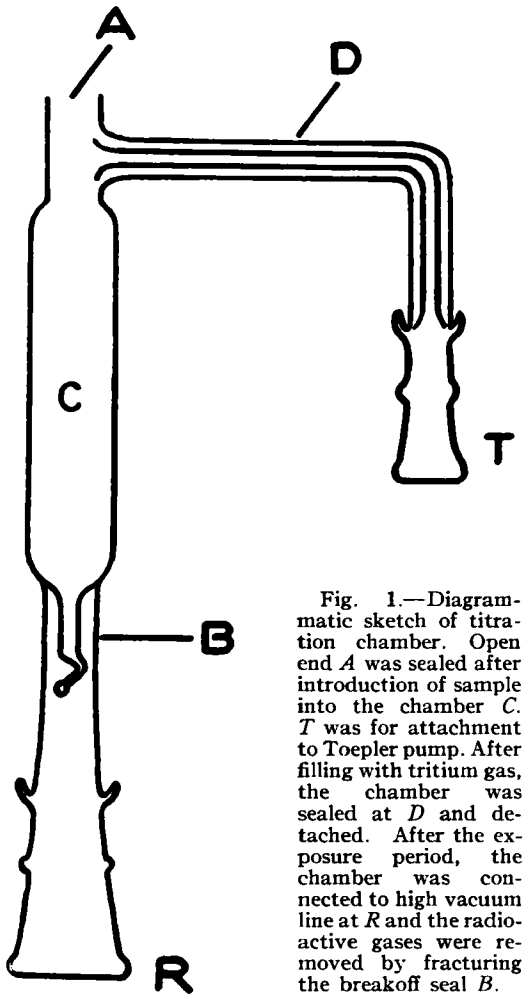

Fig. 1.—Diagrammatic sketch of titration chamber. Open end *A* was sealed after introduction of sample into the chamber *C*. *T* was for attachment to Toepler pump. After filling with tritium gas, the chamber was sealed at *D* and detached. After the exposure period, the chamber was connected to high vacuum line at *R* and the radioactive gases were removed by fracturing the breakoff seal *B*.

residue was recrystallized[1] from dry acetone at −80° to eliminate acetone soluble radioactive impurities. The recrystallized tritio-cholecalciferol (approximately 100 mg.) was dissolved in 150 ml. of peroxide-free ethyl ether and the ether solution was washed successively with three 50-ml. portions of ice-cold water to remove any hydroxylic protium exchangeable activities. The solution was dried with anhydrous sodium sulfate, filtered, and taken to dryness at room temperature in a rotatory evaporator. Whenever the residue was not completely clear, it was azeotropically dried by repeated evaporation with 20 ml. of a mixture of equal volumes of benzene and absolute ethanol. The clear residue was then taken up in 50 ml. of *n*-hexane for purification by column chromatography.

Approximately 10 to 15 mg. of the partially purified tritio-cholecalciferol in *n*-hexane was applied to a chromatographic column, 1.2 cm. in diameter and 15 cm. in height, prepared by pouring a slurry of 12 Gm. of 60 to 80-mesh Florisil in *n*-hexane into the chromatographic tube containing the same solvent. The column was kept at 15° by a water jacket to prevent solvent evaporation and thus eliminate formation of bubbles along the adsorbent bed. After loading, the column was rinsed with 100 ml. of *n*-hexane followed by elution with 400 ml. of 10% ethyl ether in *n*-hexane. The flow rate of the eluent was adjusted to 2 to 3 ml. per minute with a Teflon plugged stopcock at the lower end of the column. The eluent was collected in 10-ml. fractions. Concentration of tritio-cholecalciferol in the eluent was measured with Nield's reagent (12) against a known standard.

Purity of the tritiated cholecalciferol was ascertained by thin-layer chromatography on 250 μ-thick silica gel plates using a mixture of chloroform: methanol:water (10:50:10 by volume) as solvent. Color development was effected by spraying the plates with 20% antimony pentachloride in chloroform (13). Appearance of a pink color changing to dark brown indicated the presence of vitamin D_3.

The processes of recrystallization from acetone at −80° and column chromatography on Florisil were repeated until a constant specific activity was achieved.

Radioactivity Measurement.—Radioactivity of all samples was measured in a liquid scintillation spectrometer using 10 ml. of 0.3% diphenyloxazole in toluene as scintillator as previously reported (14). For samples in aqueous solution a scintillator consisted of 5 ml. of the above solution plus 5 ml. of a dioxane solution containing 7 Gm. diphenyloxazole, 50 mg. POPOP (2,2′-*p*-phenylenebis (5-phenyloxazole)), and 50 Gm. of naphthalene to 1 L. of purified dioxane.

The amount of the label in thin-layer chromatograms of tritio-cholecalciferol was determined by transferring the silica gel in narrow sections from the chromatogram into counting vials and assaying the radioactivity by liquid scintillation counting.

Absorption Spectrum.—The infrared spectra of unpurified tritio-cholecalciferol, nonradioactive vitamin D_3, and an unidentified decomposition product were obtained with a Beckman IR-5 infrared spectrophotometer using the KBr disk method. The ultraviolet spectra of these compounds were deter-

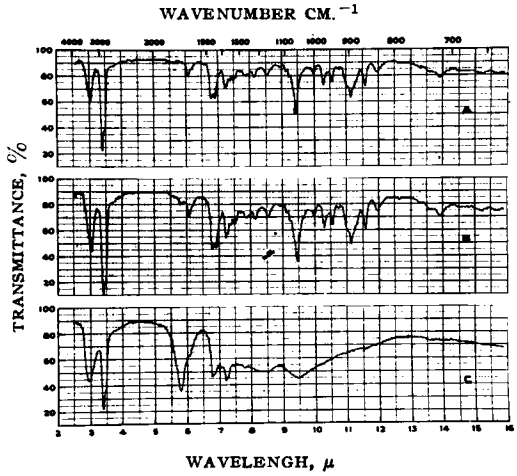

Fig. 2.—Infrared absorption spectra of cholecalciferol (*A*), unpurified tritio-cholecalciferol (*B*), and an unidentified decomposition product (*C*).

[1] The presence of moisture occasionally caused vitamin D_3 to separate as a viscous oil at this temperature in which case the supernate was removed by aspiration.

mined with a Cary model 11 recording spectrophotometer, using an ethanolic solution of 2 mg.% concentration.[2]

RESULTS AND DISCUSSION

The average β energy of tritium is 5.5 kev with a half-thickness value of 0.031 mg./cm.[2] in hydrogen (15) and a maximum range of 6 μ in water (16). At the prevailing pressure in the tritiation chamber, complete dissipation of the decay energy of tritium within the container is certain. The radiation dose to the cholecalciferol crystals, which occupied only a fraction of the total volume of the chamber, was estimated to be a minimum of approximately 2.4×10^7 rads. Apparently at $-198°$, this dose level did not cause excessive radiation damage as indicated by the identity of the infrared spectrum of the unpurified tritio-cholecalciferol (*B*) and that of an authentic nonradioactive standard (*A*) (Fig. 2). It should be noted, however, that the former contains a small absorption band at 5.8 μ, characteristic of carbonyl groups.

The purity of the tritio-cholecalciferol was ascertained by thin-layer chromatography on silica gel. The R_f value and the contour of the vitamin D_3 spot after solvent irrigation varied not only with the quantity of vitamin D_3 but also with the polarity of the solvent used for spotting. For instance, the spot obtained from a solution of vitamin D_3 in *n*-hexane or in petroleum ether (b.p. 40–60°) was more compact in shape and faster in movement as shown by an increased R_f value than that obtained from a solution in acetone, probably because of differences in ease of evaporation of the solvents and in the intrinsic solubility of vitamin D_3 in them. The R_f value of vitamin D_3 also varied inversely with the quantity of material being chromatographed on the thin-layer silica gel plate in the region of 5 to 40 mcg. regardless of the spotting solvent. In this concentration range, the vitamin D_3 spot tended to migrate nearer to the solvent front. At high concentrations ranging from 60 to 200 mcg., migration of the spot remained practically constant (R_f value: 0.651 ± 0.017 (S.D.) for hexane; 0.791 ± 0.012 (S.D.) for acetone). For routine assay, this uncertainty of the R_f value may be overcome by chromatographing spots of graded material concentration or by comparison with spots co-chromatographed with a known standard.

Distribution of tritium label in the unpurified tritio-cholecalciferol was studied on a 10-mg. sample. The results are shown in Table I. The labile tritium activity exchangeable with protium atoms on equilibration with acetone, water, and ether amounted to 73% of the initial activity. Purification by recrystallization from acetone at $-80°$ which was carried out with the bulk tritiated vitamin D_3 was neglected in this case because it would entail an excessive loss of material. Recovery of radioactivity from column chromatography on Florisil was about 71% although material recovery using a nonradioactive standard was 92%. Apparently the adsorbent at the top of the chromatographic column retained some radiation decomposition products as a yellowish band; this band was intensely radioactive and could not be eluted by acetone.

Radioactivity eluted by acetone represented approximately 53% of the label initially applied to the column. This acetone eluable fraction was found by means of thin-layer chromatography to coincide with a fast running spot which frequently appeared after long storage of known solutions of nonradioactive vitamin D_3 and which had a R_f value of approximately 1. The infrared spectrum of this compound was shown as *C* in Fig. 2. Loss of fine structure in the region of 7 to 14 μ and the presence of carbonyl stretching band at 5.8 μ, together with the minimal absorption in ultraviolet region of an ethanolic solution (2 mg.%) serve to indicate the absence of the tri-ene conjugation in the molecule. However, no attempt was made to identify this compound.

Tritio-cholecalciferol was eluted with 10% ether in *n*-hexane. The specific activity of the eluent fractions along the ascending and descending limbs of the band was found to be practically identical within experimental error, thus indicating the absence of radioactive congeners of high specific activity as possible contaminants. However, to insure a high degree of radiopurity, only the peak fractions of the tritio-cholecalciferol band were pooled for further purification. In this manner, by successive purification processes, including one recrystallization from acetone at $-80°$ and three chromatographic passages on Florisil column, a preparation of pure tritiated vitamin D_3 with a specific activity of 216 μc./mg. was obtained. The adequacy of this compound as a tracer was verified by biological studies (17) which yielded results similar to those found for nonradioactive vitamin D_3.

Condition of the adsorbant for column chromatography was found to be essential for successful separation of the tritio-cholecalciferol from its contaminants. Florisil activated at 260° was found to give better results than that deactivated with 6% water or neutral alumina (Brockmann activity III). The latter two adsorbants yielded vitamin D_3 fractions contaminated with trace of the fast-moving impurity described above, possibly due to artifact of decomposition on column. Purification of tritio-cholecalciferol by preparative thin-layer chromatography on silica gel is also feasible, provided that oxidation and photodecomposition are avoided.

TABLE I.—TRITIUM INCORPORATION AND DISTRIBUTION IN UNPURIFIED TRITIO-CHOLECALCIFEROL

		Tritium Activity
I.	Incorporation after exposure of 1.95×10^3 curie-days	2.7 mc./mg.
II.	Loss after equilibration with (%)	
	Acetone	66.6
	Water	5.6
	Ethyl ether	<1.0
III.	Distribution in chromatographic eluents (%)	
	n-Hexane	2.3
	10% Ether in *n*-hexane	15.3
	Acetone	53.4
	Activity remained on column (by subtraction)	29.0

[2] The measurement was performed by M. K. Hrenoff of the Spectrographic Laboratory, School of Pharmacy, University of California, San Francisco.

When a sample of vitamin D_3 was exposed to tritium gas at room temperature, no useful product was isolated, indicating that the use of low temperature (−198°) for labeling by exposure to tritium gas is of marked advantage since the formation of radioactive impurities is decreased. Because β labeling is due to formation of excited and ionized molecular species (18) which require energy of activation for subsequent chemical reactions, it is reasonable to postulate that at −198°, the small RT value in the Boltzmann factor reduces the probability of reaction. In addition, recombination of molecular ions is facilitated because their diffusion in solids is restricted as a result of an enhancement of the reaction cage effect at −198°.

The validity of the latter factor is supported by the work of Wenzel, *et al.* (19), who noted an increase in specific activity as well as fewer radioactive impurities after gas exposure labeling of charcoal-adsorbed compounds compared to the nonadsorbed control compounds. It is probable that either charcoal adsorption or low temperature (−198°) brings about a common effect, *i.e.*, a passive rigidity of molecular configuration and a restricted diffusion of molecular ions; both of these effects tend to diminish radiation decomposition and to increase substitution by tritium during exposure.

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Kinetics of the Metabolism of Acetaminophen by Humans

By EINO NELSON and TADASHI MORIOKA

The kinetics of the metabolism of acetaminophen to its sulfuric and glucuronic acid conjugates has been studied in normal adult humans. In post-absorptive and post-equilibrative times the elimination process was found to be first order with a mean half-life of 1.95 hours with a range of 1.62 to 2.83 hours in nine tests using a five-subject test panel. Previously published data on pain threshold elevation resulting from ingestion of acetaminophen were examined during times following maximum elevation. Decay of pain threshold elevation was shown to be an apparent first-order process with a half-life of 0.42 hours. This half-life was less than one-fourth the half-life for metabolism, indicating that poor correlation exists between pharmacological activity and body level of acetaminophen, even though a kinetic relationship may exist between these quantities.

THE ANALGESIC DRUG, acetaminophen (4′-hydroxyacetanilide), is known to be metabolized nearly completely after administration to humans. Information on the metabolic fate of this drug in humans has been summarized by Williams (1). About 3% of an oral dose is excreted unchanged in the urine, and most of the balance of a dose can be found in the urine conjugated with sulfuric and glucuronic acids, with the latter conjugate predominating. The conjugates are formed at the 4′-hydroxy position on the molecule. Apparently the kinetics of the metabolism of this substance have not been studied previously. This communication reports the results of a pharmacokinetic study of the metabolism and excretion of acetaminophen and discusses the results obtained in terms of the physiological and pharmacological factors involved.

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EXPERIMENTAL

Subject and Test Procedure.—One-gram doses of drug grade acetaminophen (finely powdered by

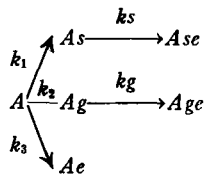
grinding in a mortar with a pestle and filled in hard gelatin capsules) were ingested in the morning on overnight-fasted stomachs by adult humans apparently in normal health. No food was taken until at least 1 hour after ingestion of the drug. The designation, sex, age, weight in kilograms, and body surface in sq. m. of the test subjects, respectively, were E-M-43-77-2.50, V-M-22-68-1.84, T-M-29-60-1.60, G-M-28-59-1.70, and S-F-37-57-1.62. Body surface areas were obtained from a nonogram (2) relating this quantity to height and weight. For determining blank excretion, urine specimens over a timed interval were obtained immediately prior to ingestion of the doses. Following ingestion of the doses, collection of urine at appropriate times was quantitative during the active course of the experiments. Nine experiments were made using the five-subject test panel.

Analytical Method.—The urines were assayed in triplicate for free acetaminophen by the method of Brodie and Axelrod (3) and in triplicate for total acetaminophen by a modification of the method described in the same publication. For determination of total acetaminophen, hydrolysis was effected by making suitably diluted urine 6 *N* in titer with hydrochloric acid and heating for 1.5 hours at 100°. One complete set of urines was assayed by both the modified and original method. Cumulative excretion of total drug was 942 mg. by the original method and 975 mg. by the modified method. The precision of the modified method was of the same order as the original method. The acetaminophen used to construct the standard curves was from the same lot used in the *in vivo* experiments.

RESULTS AND DISCUSSION

Cumulative free and conjugated drug excretions are listed in Table I.

Excretion Kinetics.—It was assumed that Scheme I described the kinetics of the metabolism and excretion of acetaminophen and its metabolites after absorption of a dose and attainment of apparent equilibrium between drug in blood and drug in other fluids of distribution



Scheme I

In Scheme I, A is the amount of acetaminophen in the body at any time; As and Ag are the amounts of acetaminophen in the form of its sulfuric and glucuronic acid conjugates, respectively, in the body at any time; Ase and Age are the amounts of acetaminophen sulfate and glucuronide ethers excreted in the urine in any time; and Ae is the amount of unchanged acetaminophen excreted in any time. The k's with number or letter subscripts are first-order rate constants with the dimensions of reciprocal time for the processes indicated. First-order processes for metabolism and urinary excretion are assumed since it has been found that this type of process describes within experimental error (in post-absorptive and post-equilibrative times) the metabolism and disposition of many drugs and foreign substances (4–15).

A preliminary examination of urinary excretion data indicated that apparent first-order excretion was being followed 2 or 3 hours after ingestion of doses. For mathematical treatment of data, a zero time shift (13, 14) of these values of time was made and cumulative amounts of drug excreted corrected to agree with the new time base by subtraction of the amount excreted in the time equal to the time shift.

TABLE I.—CUMULATIVE AMOUNTS OF FREE (F) AND CONJUGATED (C) ACETAMINOPHEN EXCRETED AT VARIOUS TIMES[a]

	1 Hr.		2 Hr.		3 Hr.		4 Hr.		5 Hr.		6 Hr.		7 Hr.		8 Hr.		9 Hr.		Terminal[b]	
Subject	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
E-1	1	16	3	126	8	321	11	529	13	628	15	714	16	792	17	822	17	841	21	900
E-2	6	40	9	160	15	380	21	568	24	677	26	785	27	847	..	...	28	903	28	942
E-3	7	46	11	170	18	396	25	580	28	692	31	802	33	865	..	...	35	922	36	965
E-4	..	...	21	293	24	396	30	616	34	717	38	822	40	873	..	...	42	935	44	995
E-5	8	29	20	173	28	334	33	479	36	577	38	666	42	726	43	761	45	799	48	915
V	2	41	11	285	22	492	30	665	36	805	44	926	45	967	47	1010	49	1040	51	1081
G	0	100	6	287	11	490	13	645	15	747	17	827	18	865	19	900	20	933	21	969
T	5	0	9	169	13	332	16	463	19	558	22	628	..	...	24	710	..	...	35	904
S	..	...	13	205	..	...	24	586	26	716	27	806	..	...	..	...	28	911	29	942

[a] All data expressed as mg. of free drug. [b] Between 12 and 24 hours depending on a given subjects' sampling schedule.

TABLE II.—RATE CONSTANTS AND HALF-LIVES FOUND FOR THE METABOLISM AND EXCRETION OF ACETAMINOPHEN BY HUMANS[a]

Test Subjects	E-1	E-2	E-3	E-4	E-5
Rate constant (K), $hr.^{-1}$	0.400 (2.69×10^{-2})	0.385 (3.77×10^{-2})	0.394 (3.83×10^{-2})	0.427 (4.36×10^{-2})	0.275 (8.37×10^{-3})
Half-life, hr.	1.73 (1.14×10^{-1})	1.80 (1.79×10^{-1})	1.76 (1.85×10^{-1})	1.62 (1.60×10^{-1})	2.52 (3.60×10^{-2})
Test Subjects	**V**	**G**	**T**	**S**	**Mean**
Rate constant (K), $hr.^{-1}$	0.385 (4.58×10^{-2})	0.391 (1.90×10^{-2})	0.245 (1.28×10^{-2})	0.408 (3.73×10^{-2})	0.368 (5.60×10^{-2})
Half-life, hr.	1.85 (2.44×10^{-1})	1.77 (8.37×10^{-2})	2.83 (1.61×10^{-1})	1.70 (1.57×10^{-1})	1.95 (2.26×10^{-1})

[a] Standard deviations shown in parentheses.

In the model above it was further assumed that excretion of the metabolites of acetaminophen would be rate limited by the formation steps ($k_e \gg k_1$ and $k_g \gg k_2$).

On the basis of the considerations discussed in the preceding paragraphs, the following differential equations describe the several processes shown in the model

$$\frac{dA}{dE} = -KA \qquad \text{(Eq. 1)}$$

$$\frac{dAse}{dt} = k_1A \qquad \text{(Eq. 2)}$$

$$\frac{dAge}{dt} = k_2A \qquad \text{(Eq. 3)}$$

$$\frac{dAe}{dt} = k_3A \qquad \text{(Eq. 4)}$$

In Eq. 1 $K = k_1 + k_2 + k_3$. Equation 1 when integrated with constant of integration evaluated at zero time, where A° is the amount of acetaminophen in the body at zero time gives

$$A = A^\circ \, exp\,[-Kt] \qquad \text{(Eq. 5)}$$

Substitution in Eq. 2 of the value for A given in Eq. 5, and integration of the resulting expression with constant of integration evaluated at zero time under the condition that no metabolite was excreted gives

$$Ase = \frac{k_1A^\circ}{K}(1 - exp\,[-Kt]) \qquad \text{(Eq. 6)}$$

By a procedure identical with the immediately preceding one, Eq. 3 becomes

$$Age = \frac{k_2A^\circ}{K}(1 - exp\,[-Kt]) \qquad \text{(Eq. 7)}$$

and Eq. 4 becomes

$$Ae = \frac{k_3A^\circ}{K}(1 - exp\,[-Kt]) \qquad \text{(Eq. 8)}$$

In the experimental procedure adopted for following excretion of sulfuric and glucuronic acid ethers of acetaminophen, both substances were determined and the relative amounts of each form were unknown. Therefore, the combined excretion should be given by the sum of Eqs. 6 and 7

$$Am = \frac{k_1A^\circ}{K}(1 - exp\,[-Kt]) + \frac{k_2A^\circ}{K}(1 - exp\,[-Kt]) \qquad \text{(Eq. 9)}$$

In Eq. 9 Am is the amount of the sulfuric and glucuronic acid conjugates of acetaminophen excreted in the urine at any time. After a sufficiently long time corresponding to the time that only negligible excretion of metabolites occurs, where Am° is total amount of metabolites excreted after zero time, Eq. 9 reduces to

$$Am^\circ = \frac{k_1A^\circ}{K} + \frac{k_2A^\circ}{K} \qquad \text{(Eq. 10)}$$

If the value of the constants on the right-hand side of Eq. 10 are substituted in Eq. 9, the latter equation becomes

$$Am = Am^\circ(1 - exp\,[-Kt]) \qquad \text{(Eq. 11)}$$

Thus a relatively simple expression describes excretion of the metabolites of acetaminophen. Equation 11 was used in the form

$$\log_e(1 - Am/Am^\circ) = -Kt \qquad \text{(Eq. 12)}$$

After the shifts in zero time, at each of the data point times except the last ones, values of K were calculated, using the experimental data and Eq. 12. The values were then averaged to give a mean value of this quantity for a given experiment. These values were then divided into 0.693 to give the rate of the processes in terms of a half-life ($t^1/_2$). Because K represents the sum of all rate constants determining the rate of removal of acetaminophen

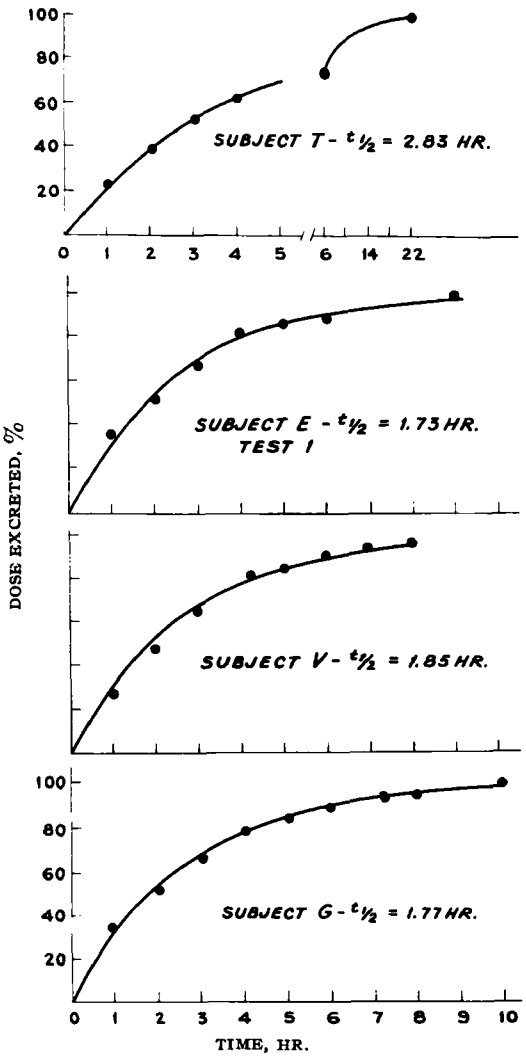


Fig. 1.—Cumulative excretion curves of acetaminophen's metabolites following oral ingestion of 1-Gm. doses of acetaminophen. Curves shown are theoretical for excretion after the time taken as zero time. Plotted points are experimentally observed values of cumulative excretion at the same times. Each curve is labeled with the test subject's designation and half-life for disposal of the drug from the body in a given experiment.

TABLE III.—RATE CONSTANTS AND HALF-LIVES FOUND FOR THE URINARY EXCRETION OF FREE ACETAMINOPHEN BY HUMANS

Test Subject	E-1	E-2	E-3	E-4	E-5	V	G	T	S	Mean
Rate constant, (k_3), hr.$^{-1}$ × 10^3	9.12	11.1	14.2	18.5	13.7	17.3	8.29	9.11	11.3	12.5
Half-life, hr.	76.0	62.4	48.8	37.4	50.6	40.0	83.6	76.1	61.3	59.6

from the body, the half-life so found represents the half-life for acetaminophen's disposal by the body.

Values of the rate constants, K, and half-lives found in all the experiments are given in Table II. To show that Eq. 12 did describe the excretion of acetaminophen's metabolites within experimental error, the mean values of K were substituted into Eq. 12 and theoretical excretion curves calculated. Some of these are shown in Fig. 1. The experimental points are included on the same plots, and it is shown that there is good agreement between theoretically predicted and experimentally observed cumulative acetaminophen metabolite excretion.

Free drug excretion was in such small amounts that it was not considered practical to fit the data to Eq. 8. The rate constant k_3 is deducible, however, from other information obtained in the experiments. At the time which corresponds to the end of the active course of experiments, Eq. 8 reduces to

$$Ae^\circ = \frac{k_3 A^\circ}{K} \qquad \text{(Eq. 13)}$$

where Ae° is the total amount of drug excreted as unchanged material. At this time Eq. 10 also holds which may be rearranged to give

$$A^\circ = \frac{KAm^\circ}{k_1 + k_2} \qquad \text{(Eq. 14)}$$

or

$$A^\circ = \frac{KAm^\circ}{K - k_3} \qquad \text{(Eq. 15)}$$

since $K = k_1 + k_2 + k_3$. If Eq. 13 is solved for A°, equated to Eq. 15, and the resulting expression rearranged, the value of the rate constant for excretion of free acetaminophen is

$$k_3 = \frac{Ae^\circ K}{Ae^\circ + Am^\circ} \qquad \text{(Eq. 16)}$$

All quantities on the righthand side of Eq. 16 are known; hence the value of k_3 may be obtained. In the present experiments the mean value was found to be 12.5×10^{-3} hr.$^{-1}$ The individual values of these constants are listed in Table III. If the relative amounts of drug conjugated with sulfuric and glucuronic acids are known, it is easy to show that the rate constants k_1 and k_2 would be, respectively

$$k_1 = \frac{Ase^\circ K}{Ae^\circ + Am^\circ} \qquad \text{(Eq. 17)}$$

and

$$k_2 = \frac{Age^\circ K}{Ae^\circ + Am^\circ} \qquad \text{(Eq. 18)}$$

where Ase° and Age° are the total amounts of drug found conjugated with sulfuric acid and glucuronic acids, respectively.

Pain Threshold Elevation and Kinetics of Metabolism.—It is of interest to compare the results of the present kinetic study with the kinetics of the analgesic effect of acetaminophen. Elevation of pain threshold following oral administration of 0.325-Gm. doses of drug to adult humans has been studied (16). After the maximum elevation in pain threshold, which occurred about 2.5 hours after administration of the doses, a rapid decay in elevation occurred. When values of elevation after the maximum were plotted as a function of time using semilogarithmic paper, a straight line resulted which indicated an apparent first-order process for decline in activity. This is shown in Fig. 2. Other instances of apparent first-order decay in pharmacological effects have been discussed by Swintosky (17). It is shown from Fig. 2 that the half-life for acetaminophen's activity is about 0.42 hours. In the paper where the data used to constant Fig. 2 were reported (16), it was stated that pain threshold elevation paralleled acetaminophen serum levels observed in another study (18). Examination of these data (which were limited) indicated that that decay in free acetaminophen levels in the post-absorptive phases had a half-life of approximately 1.9 hours. This half-life is more than four times as long as that which described decay in pain threshold elevation. The serum half-life of 1.9 hours compares favorably in magnitude to the mean half-life found in the

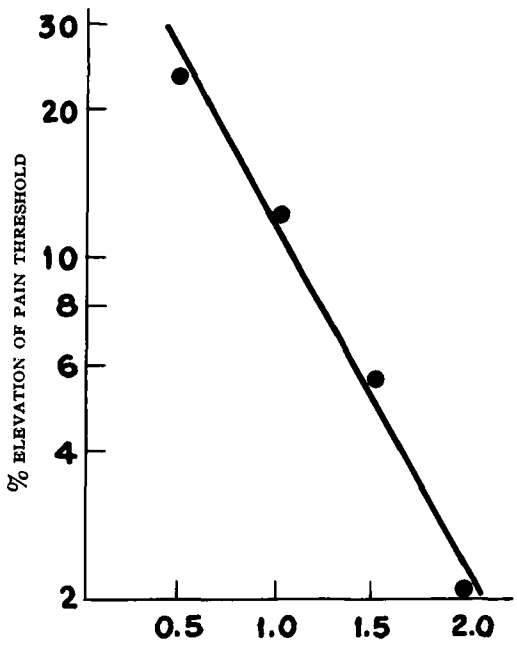


Fig. 2.—Illustrating first-order decay in pain threshold elevation after maximum observed elevation in threshold after oral ingestion of acetaminophen. Data for graph are taken from Flinn and Brodie (16).

present study of 1.95 hours. It would appear that disappearance of acetaminophen's pharmacologic effects bears little relationship to the rate with which this drug is metabolized and excreted, even though a kinetic relationship may exist between these quantities.

Biological Variations and Body Surface Area of Test Subjects.—Expected biological variation was found in the individual values of either the half-life or rate constant for disposal of acetaminophen. Since the rates of other physiological processes are sometimes related to body surface area, it was of interest to examine the present results to determine whether these were factors in causing the variation observed. There was no evidence of a relationship between surface area and rate constant when these quantities were plotted.

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Aryl Indolizines I

Synthesis and Properties of Some Phenylindolizines

By VINCENT S. VENTURELLA†

Several aromatically substituted indolizines were prepared for possible psychotropic activity utilizing the Tschitschibabin method, and the susceptibility of the nucleophilic position toward benzoyl chloride was established. The properties of the prepared compounds indicated that those with an aromatic halide are not susceptible to nucleophilic attack such as the formation of alkoxy derivatives and also that those compounds containing the *p*-nitrophenyl group could be reduced to the corresponding amino compounds only under drastic conditions not normally expected of such compounds.

THE PHYSIOLOGICAL ACTIVITY of serotonin analogs (1) and reserpine (2) suggests that the structurally similar diphenylindolizines may possess psychotropic activity. In addition, it has been reported (3) that several alkylindolizines have a convulsant activity which would tend to indicate a profound effect on the central nervous system. It is hoped that the use of aryl substituents will moderate this activity thus producing compounds having the desired effect.

It is possible to prepare substituted indolizines by methods developed by Tschitschibabin (4), Barrett (5), or Scholtz (6), the former being most desirable because of ease of formation, good yields, and availability of starting materials. A modification of the method of Moser and Bradsher (7) permitted easy quaternization of the corresponding aryl pyridine with a substituted phenacyl halide to produce a solid product (Table I) in the absence of solvent followed by cyclization according to the scheme shown in Eq. 1.

When 2-parachlorobenzylpyridine was employed, the conditions necessary for the quaternization forced a spontaneous cyclization to occur, thus making it difficult to isolate these intermediates. Considerable decomposition of the tar base, which was always present in excess, was evident in the quaternization step either with 2-benzylpyridine or the *p*-chloro analog. The indolizines prepared are summarized in Table II.

The resonating system of the indolizines in the active state results in an alternating system of electron density (8) which causes nucleophile activity at position 1 or at position 3 if the former is substituted, leading to facile attack by electrophilic reagents. Thus treat-

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TABLE I.—SUBSTITUTED 2-BENZYLPYRIDINIUM ACETOPHENONE BROMIDES

Compd.	Condensation Temp., °C.	Time, min.	Leaching Solv.	Recryst. Solv.	M.p., °C.	Yield, %	Anal., % Calcd. C	H	N	Found C	H	N
IA	40	20	EtOH	Hot H_2O	236–238, dec.	54	53.78	3.81	3.14	53.48	3.80	3.40
IB	50	20	MeOH	Aq. MeOH (cold) 10%	211–212.5, dec.	85	58.10	4.12	6.78	57.78	4.18	7.00
IC	60	35	EtOH (cold)	Aq. MeOH (hot) 50%	248–251, dec.	48	70.30	4.96	3.16	70.32	4.91	3.13

TABLE II.—ARYL INDOLIZINES

Compd.	Reflux Time, hr.	Recryst. Solv.	M.p., °C.	Yield, %	Anal., % Calcd. C	H	N	Found C	H	N
IIA	3	Aq. MeOH (hot)	138 to 139.5	89[a]	69.00	4.02	4.02	68.63	3.98	4.19
IIB	2	Boiling Me_2CO	166–167, dec.	94[a]	76.50	4.46	8.92	75.63	4.62	9.00
IIC	2	Boiling EtOH	196–197, dec.	93[a]	90.25	5.79	4.05	89.72	5.78	4.03
IID	5	Me_2CO-H_2O	178–180, dec.	63[b]	62.99	3.41	3.68	62.69	3.55	3.66
IIE	7	Me_2CO-H_2O	214–216	49[b]	68.97	3.74	8.05	68.99	3.69	8.05
IIF	8	Me_2CO-H_2O	195–196	30[b]	82.32	4.75	3.69	82.41	4.99	3.60

[a] Based on pyridinium salt. [b] Based on starting substituted acetophenone.

ment with benzoyl chloride gave fair to good yields of the corresponding benzoyl derivatives by a modification of the procedure of Holland (9). The benzoyl compounds are peculiar in their lack of reactivity toward carbonyl reagents, but are said to form 2,4-dinitrophenylhydrazones readily (10). However, the compounds prepared in the present study formed these derivatives in poor yields and only after several hours of heating. In addition, the prepared benzoyl compounds have an infrared spectrum which would tend to preclude the presence of the carbonyl group. The effect of adjacent aromatic groups on the wavelength of absorption (11) coupled with reports that alkyl formyl derivatives (12) absorb at 6.1 μ tends to substantiate the fact that the carbonyl compounds of the present study had an absorption at 6.3 to 6.4 μ.

Anomolus behavior of the aryl indolizines prepared was found to exist in two instances. First, a fair degree of basicity should be evident because many of literature compounds form salts with mineral acid (10). In all cases of the present study, the compounds could be dissolved in concentrated mineral acid, but quantitatively precipitated upon dilution with even minute amounts of water. This anomoly is most logically attributed to the presence of multiple resonating systems which inactivate the available electrons on the tertiary nitrogen. Although a report (13) indicates that the presence of an aryl nitro group retards hydrolysis of 1-acyl groups, it was found that if a *p*-nitrophenyl group and a *p*-chlorophenyl group were at positions 2 and 3, respectively, the benzoyl derivative could not be isolated. The evidence discounts the possibility that hydrolysis occurs during reaction or processing. The only other explanation for this result is that the presence of a *p*-chlorophenyl group in the 3-position increases the neighboring group neutralizing effect of the resonance form of the *p*-nitrophenyl group, thereby preventing a nucleophilic center from being established.

Preliminary studies on the reactivity of the *p*-chlorophenyl group indicated that it has a low order of reactivity because it failed to undergo nucleophilic substitution in refluxing methanolic sodium methoxide.

EXPERIMENTAL[1]

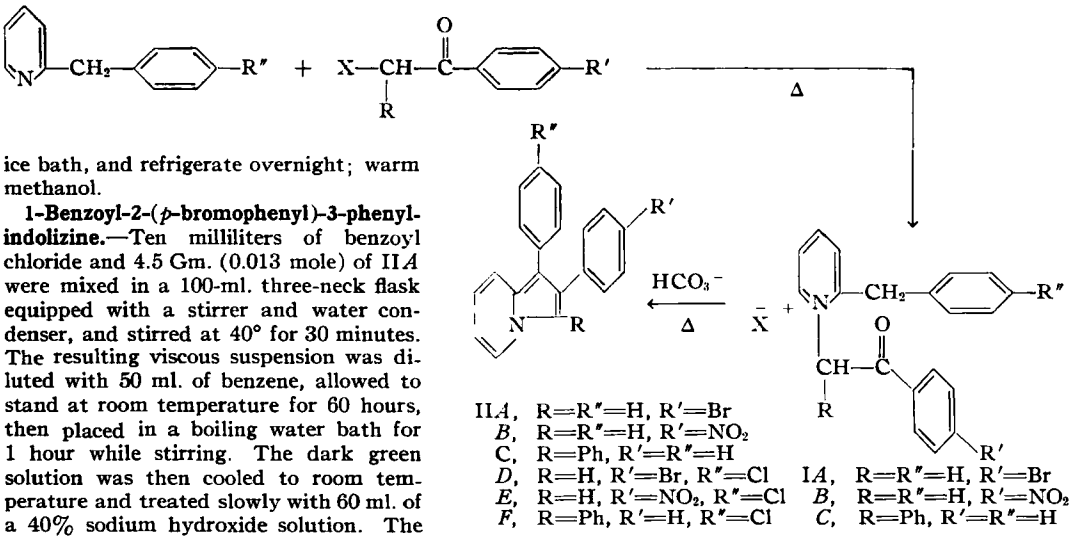
The first two parts of the Experimental are general procedures, the details of which are presented in Tables I and II.

2-Benzylpyridinium Acetophenone Bromides.—The appropriate bromoacetophenone was mixed with 3 equiv. of 2-benzylpyridine and warmed to the required temperature on a water bath with constant agitation until solidification occurred. The resulting product was refrigerated for 2 days. The solid was then fragmented, leached, removed by filtration, and recrystallized.

Cyclodehydration of the Pyridinium Salts.—The pyridinium salt was placed in a three-neck flask equipped with a stirrer and water condenser and treated with a saturated solution of sodium bicarbonate (20 Gm./500 ml.) and 10 ml. of ethanol. The mixture was refluxed with constant stirring for the required period of time. The resulting slurry was filtered hot, the solid washed several times with hot water, dried by suction, and recrystallized.

Quaternization with 2-*p*-Chlorobenzylpyridine.—In each case, the substituted pyridine was mixed with the bromoacetophenone and heated as described for the 2-benzylpyridinium salt. The temperature, time of condensation, and the leaching solvent for the *p*-chloro analogs of compounds *IA*, *IB*, and *IC*, respectively, were as follows: 50° for 10 minutes, stand at room temperature for 3 days; ethanol: 60° until reddish brown and syrupy, shake until solid, and refrigerate 3 days; methanol: steam bath until dark brown and syrupy, shake until solid, immediately cool in an

[1] All melting points were taken on a Fisher-Johns apparatus and are uncorrected. Microanalyses by Schwartzkopf Microanalytical Laboratories, Woodside, N. Y.



IIA, R=R″=H, R′=Br
B, R=R″=H, R′=NO2
C, R=Ph, R′=R″=H
D, R=H, R′=Br, R″=Cl
E, R=H, R′=NO2, R″=Cl
F, R=Ph, R′=H, R″=Cl

IA, R=R″=H, R′=Br
B, R=R″=H, R′=NO2
C, R=Ph, R′=R″=H

(Eq. 1)

ice bath, and refrigerate overnight; warm methanol.

1-Benzoyl-2-(*p*-bromophenyl)-3-phenylindolizine.—Ten milliliters of benzoyl chloride and 4.5 Gm. (0.013 mole) of II*A* were mixed in a 100-ml. three-neck flask equipped with a stirrer and water condenser, and stirred at 40° for 30 minutes. The resulting viscous suspension was diluted with 50 ml. of benzene, allowed to stand at room temperature for 60 hours, then placed in a boiling water bath for 1 hour while stirring. The dark green solution was then cooled to room temperature and treated slowly with 60 ml. of a 40% sodium hydroxide solution. The benzene layer was removed, washed with 60 ml. of water, and the combined aqueous layers washed with benzene until the benzene washings were colorless. The benzene layers were then dried over anhydrous sodium sulfate and evaporated under reduced pressure to a thick green syrup. The addition of 50 ml. of petroleum ether (30–60°) to the syrup caused precipitation of a greenish brown solid. The solid was removed by filtration, washed with two 50-ml. portions of petroleum ether, and suction dried to give 4.0 Gm. (68%) of a bright green powder. It crystallized from boiling ethanol as needles, m.p. 196–198°.

Anal.—Calcd. for $C_{27}H_{18}BrNO$: C, 71.68; H, 3.98; N, 3.10. Found: C, 71.48; H, 3.98; N 3.10.

2,4-Dinitrophenylhydrazone.—Red-brown scales (95% ethanol), m.p. 140–142°.

Anal.—Calcd. for $C_{33}H_{22}BrN_5O_4$: N, 11.07. Found: N, 11.52.

1-Benzoyl-2-(*p*-bromophenyl)-3-(*p*-chlorophenyl)indolizine.—Five grams (0.013 mole) of the corresponding indolizine was mixed at 60° (water bath) with 12.5 ml. of benzoyl chloride, stirred for 1 hour, diluted with 25 ml. of benzene, and kept at this temperature for 2 additional hours. After standing for 48 hours at room temperature, the green solution was diluted with 50 ml. benzene, made basic with a 40% solution of sodium hydroxide, and the liquids were separated. Processing as previously described gave a green solid which was washed twice with ice-cold acetone and suction dried to give 4.5 Gm. (70%) of a crystalline solid. Recrystallization from hot acetone gave yellowish-green flakes, m.p. 235–237°.

Anal.—Calcd. for $C_{27}H_{17}BrClNO$: C, 66.81; H, 3.51; N, 2.88. Found: C, 66.78; H, 3.76; N, 2.22.

2,4-Dinitrophenylhydrazone.—Orange-red needles (70% ethanol), m.p. 185–187°.

Anal.—Calcd. for $C_{33}H_{21}BrClN_5O_4$: N, 10.55. Found: N, 10.99.

1-Benzoyl-2-(*p*-nitrophenyl)-3-phenylindolizine.—Five grams (0.016 mole) of II*B* was mixed with 12.3 ml. of benzoyl chloride and heated on a water bath at 60° for 30 minutes, giving a dark greenish-brown syrup. Ten milliliters of benzene was added, followed by refluxing for 1 hour. The mixture was stirred at room temperature for 20 hours and allowed to stand for 3 days. Processing followed by the addition of petroleum ether (30–60°) gave a dark oil which crystallized when triturated with cold ethyl acetate. Filtration and washing with petroleum ether gave 3.5 Gm. (53%) of a green solid which recrystallized as plates from boiling ethanol, m.p. 253–257°.

Anal.—Calcd. for $C_{27}H_{18}N_2O_3$: C, 77.52; H, 4.31; N, 6.69. Found: C, 77.53; H, 4.30; N, 6.77.

2,4-Dinitrophenylhydrazone.—Orange-brown granules(ethanol), m.p. 198–201°, dec.

Anal.—Calcd. for $C_{33}H_{22}N_6O_6$: N, 14.10. Found: N, 14.79.

2 - (*p* - Aminophenyl) - 3 - phenylindolizine.—Ten grams (0.031 moles) of II*B* was placed in a 250-ml. three-neck flask equipped with a stirrer and water condenser and mixed with 9 equiv. of concentrated hydrochloric acid (28 Gm.), stirred, and diluted with water to 80 ml. The stirred suspension was then heated to 60° while 0.124 moles of granular tin was added portionwise over 1.5 hours. At the end of 5 hours of heating, the suspension was cooled, allowed to stand overnight at room temperature, and filtered. The filtrate was made alkaline with 10 *N* sodium hydroxide solution. Stirring for 1 hour, followed by filtration and water washing gave 7.2 Gm. (71%) of a tan microcrystalline material. The solid was recrystallized from hot aqueous acetone and dried at 100°, m.p. 153 to 154.5°.

Anal.—Calcd. for $C_{20}H_{16}N_2$: C, 84.51; H, 5.63; N, 9.86. Found: C, 84.59; H, 5.76; N, 9.74.

2 - (*p* - Aminophenyl) - 3 - (*p* - chlorophenyl)indolizine.—Ten grams of II*E* was reduced in the same manner as II*B* to give 4.5 Gm. (49%) of product, m.p. 165–167° after recrystallization from ethanol-water.

Anal.—Calcd. for $C_{20}H_{15}ClN_2$: C, 75.47; H, 4.72; N, 8.80; Cl, 11.01. Found: C, 74.97; H, 4.67; N, 8.57; Cl, 10.94.

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Critical *In Vitro* Factors in Evaluation of Gastric Antacids I

Polypeptide Inhibition

By SAURABH DESAI, MILO GIBALDI, and JOSEPH L. KANIG

In vitro **investigation of a wide range of antacid materials contained in various commercially available dosage forms has indicated demonstrable differences in both the degree and rate of neutralization and in buffering capacities. Results obtained by using pH-titration curves reveal that the type of dosage form exerts an influence on the antacid properties of the same chemical compound. Inhibition of antacid activity of some compounds was observed when polypeptides were added to the artificial gastric fluid. It is thus conceivable that antacid activity could be completely curtailed if similar conditions are encountered in the human system.**

ANTACIDS are the object of considerable interest in therapeutics and in pharmacological and pharmaceutical research. Because of their widespread use in the treatment of peptic ulcers, hyperacidity, and gastric distress, numerous *in vitro* investigations have been instituted to evaluate antacid activity (1–11).

The increasing popularity of antacid therapy has led to the marketing of a large number of products which are based on the principle of neutralization of excess gastric acid. These commercially available products may be broadly divided into systemic and nonsystemic preparations. According to Fuchs (12) systemic antacids are defined as those which react with hydrochloric acid in the stomach to form by-products which are readily absorbed and, in turn, induce a systemic alkalosis by shifting the buffer balance of the blood. Nonsystemic antacids are said to form by-products which are insoluble in body fluids and therefore will not induce an alkalosis.

In vivo excretion data obtained from another study currently being conducted in our laboratories offer evidence that the supposed nonsystemic antacids may induce a mild but nevertheless measurable systemic alkalosis. Furthermore, almost all of the products in this category will react with HCl to form water soluble by-products such as magnesium chloride, calcium chloride, and aluminum chloride which are equally soluble in body fluids.

Since the possibility exists that the so-called nonsystemic antacids might induce systemic effects and to avoid conceptual misunderstandings because of erroneous terminology, antacids have been classified in this investigation on an entirely different basis. A study of the currently marketed antacids indicated that all products could be divided into two major product types—effervescent and noneffervescent.

Although a number of comparative studies in recent years have been reported (1–3, 7–10, 13) regarding antacid efficiency, a lack of information still exists on comparisons between (*a*) effervescent and noneffervescent antacids, (*b*) antacid products using doses recommended by manufacturers rather than equal weights of similar products or other arbitrary levels, (*c*) the different dosage forms of the same products at the recommended dosage levels for each.

Since it has the greatest effect on results, one of the most critical *in vitro* factors is the composition of the test fluid. Almost invariably a dif-

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In vivo excretion data obtained from another study currently being conducted in our laboratories offer evidence that the supposed nonsystemic antacids may induce a mild but nevertheless measurable systemic alkalosis. Furthermore, almost all of the products in this category will react with HCl to form water soluble by-products such as magnesium chloride, calcium chloride, and aluminum chloride which are equally soluble in body fluids.

Since the possibility exists that the so-called nonsystemic antacids might induce systemic effects and to avoid conceptual misunderstandings because of erroneous terminology, antacids have been classified in this investigation on an entirely different basis. A study of the currently marketed antacids indicated that all products could be divided into two major product types—effervescent and noneffervescent.

Although a number of comparative studies in recent years have been reported (1–3, 7–10, 13) regarding antacid efficiency, a lack of information still exists on comparisons between (*a*) effervescent and noneffervescent antacids, (*b*) antacid products using doses recommended by manufacturers rather than equal weights of similar products or other arbitrary levels, (*c*) the different dosage forms of the same products at the recommended dosage levels for each.

Since it has the greatest effect on results, one of the most critical *in vitro* factors is the composition of the test fluid. Almost invariably a dif-

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TABLE I.—ANTACID PRODUCTS AND INGREDIENTS EVALUATED

Code Designation	Dosage Form	Recommended Dose[a] Min.	Max.	Composition
A	Effervescent tablet	1	2	Sodium bicarbonate, citric acid, monocalcium phosphate
B	Effervescent powder	1	...	Sodium bicarbonate, sodium potassium tartrate, tartaric acid
C	Effervescent granules	1	2	Sodium bicarbonate, potassium bicarbonate, citric acid, tartaric acid, calcium lactate, sodium phosphate, magnesium sulfate
D	Tablet	2	4	Aluminum hydroxide, magnesium hydroxide
D-L	Liquid	2	4	1 teaspoonful of D-L = 1 tablet of D
E	Tablet	2	4	Magnesium hydroxide, aluminum hydroxide
E-L	Liquid	2	4	1 teaspoonful of E-L = 1 tablet of E
F	Tablet	1/2	1	Aluminum hydroxide
F-L	Liquid	1	2	1 teaspoonful of F-L = 1/2 tablet of F
G	Tablet	1	2	Aluminum hydroxide, magnesium hydroxide
G-L	Liquid	1	2	1 teaspoonful of G-L = 1 tablet of G
H	Tablet	2	4	Magnesium trisilicate, aluminum hydroxide
H-L	Liquid	1	4	1 teaspoonful of H-L = 1 tablet of H
I	Tablet	2	4	Magnesium hydroxide
I-L	Liquid	1	3	Magnesium hydroxide[b]
J	Tablet	1	2	Calcium carbonate, glycine
J-L	Liquid	1	...	Calcium carbonate, glycine[b]
K	Tablet	2	3	Sodium bicarbonate, calcium carbonate, bismuth subcarbonate, magnesium carbonate, magnesium oxide, papain
L	Tablet	6	...	Sodium bicarbonate, charcoal, ginger, capsicum.
M	Tablet	1	2	Dihydroxy aluminum-sodium carbonate
N	Tablet	1	4	Calcium carbonate, magnesium carbonate, magnesium trisilicate
O	Tablet	1	4	Aluminum hydroxide, magnesium oxide, sodium lauryl sulfate
P	Tablet	1	2	Aluminum hydroxide, calcium carbonate, magnesium peroxide

[a] Expressed as number of tablets in the case of tablets; number of teaspoonfuls (5 ml.) for liquids or granules. [b] No equivalency between whole dosage units of liquid and tablet forms is available.

ferent test fluid has been proposed for each of the evaluative procedures reported in the literature (6). In 1953, Brindle (5) found that the results obtained from *in vitro* evaluations of antacids depended upon the presence or absence of pepsin and peptone in the test fluid. Others have stated (14, 15) that such materials have little effect on the results. Recently, Beekman (16) and Newey (17) have confirmed Brindle's observations and have shown that pepsin and peptone exhibit a marked inhibitory effect on the *in vitro* activity of dried aluminum hydroxide gel. The effect of polypeptides has not, however, been evaluated with other antacids which are formulated with calcium or magnesium compounds or with effervescent antacids. Furthermore, the mechanism of such an inhibition has never been elucidated.

The objectives of this study were therefore (*a*) to compare the evaluation of the basic types of marketed antacids and the different dosage forms of individual antacid preparations, and (*b*) to investigate the role of polypeptides on *in vitro* antacid activity.

EXPERIMENTAL

Volume of Test Fluid.—According to Fuchs (12) the human stomach contains the equivalent of approximately 50 ml. of free 0.1 *N* HCl shortly after a meal. He also concludes that an additional 240 ml. of 0.1 *N* HCl is secreted within 2 hours thereafter. Based on these observations it was decided to employ 50 ml. of 0.1 *N* HCl as the starting volume in all neutralization determinations. When buffering capacities of an antacid were evaluated, 0.1 *N* HCl was added to this volume at the rate of 2 ml./minute.

TABLE II.—INDIVIDUAL ANTACID COMPONENTS EVALUATED

Code Designation	Material	Quantity Evaluated
Q	Sodium bicarbonate U.S.P.	1 Gm.
R	Sodium citrate U.S.P.	1 Gm.
S	Dried aluminum hydroxide gel U.S.P.	1 Gm.
S-L	Aluminum hydroxide gel, 10%	10 ml.
T	Magnesium trisilicate U.S.P.	1 Gm.
T-L	Magnesium trisilicate gel, 10%	10 ml.
U	Calcium carbonate U.S.P.	1 Gm.
U-L	Calcium carbonate gel, 10%	10 ml.
V	Magnesium oxide U.S.P.	1 Gm.
W	Magnesium carbonate U.S.P.	1 Gm.

TABLE III.—RATES OF NEUTRALIZATION AND MAXIMUM pH ATTAINED BY MINIMUM RECOMMENDED DOSAGES OF COMMERCIAL ANTACIDS

Code Designation	Minutes Required to Attain pH 4	Max. pH Reached
A	< 0.5	6.7
B	< 0.5	5.3
C	< 0.5	9.1
D	2 (<1)[a]	6.4
D-L	< 0.5	4.9
E	2 (1)	7.7
E-L	2	5.0
F	...	3.8
F-L	...	3.9
G	...	3.9
G-L	8	4.4
H	17 (1.5)	7.4
H-L	13	6.0
I	< 2 (<0.5)	8.9
I-L	< 0.5	8.9
J	< 2 (<0.5)	7.6
J-L	< 1	7.7
K	< 0.5	8.6
L	1 (<0.5)	9.2
M	...	3.8
N	22 (4)	7.8
O	25 (3)	5.2
P	4 (4)	7.4

[a] Figures in parentheses indicate values for crushed tablets.

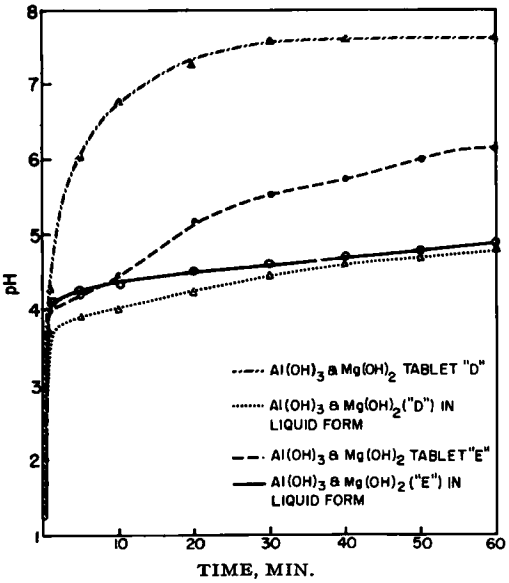

Fig. 1.—Rate of neutralization and maximum pH attained with minimum equivalent doses of two dosage forms of two commercial antacids in 50 ml. 0.1*N* HCl.

Range of Antacid Activity.—Lack of agreement exists regarding the pH range required for antacid effect. These have been reported as ranges of 2 or above, 3 or above, and from 4 to 5.5. Dale and Booth (13) have stated, "despite this apparent disagreement it is likely that all are correct since for certain clinical purposes a final pH range of from 4 to 6 may be desirable while for others a pH of 2 to 3 may suffice." These authors have advocated that antacids could be considered as being effective as long as the pH of the stomach contents remains above that of normal gastric fluid which is considered to be about 1.5 to 2. In view of these considerations, a return to a pH of 2 was considered as the end point of all buffering capacity determinations.

Selection of Antacids.—The commercial antacid products and the antacid components utilized in this study are tabulated and coded in Tables I and II. These were selected as being representative of the various types of antacids and the variety of mixtures of antacid materials currently employed.

Procedures

A. Rate of Neutralization.—The recommended minimum dose of a commercial antacid was added to a 400-ml. beaker containing 50 ml. of 0.1 *N* HCl. When the dosage form tested was a tablet, it was evaluated in both crushed and uncrushed form. The test mixture was maintained at a constant temperature of 37 ± 0.5° by immersing the beaker in a constant temperature water bath. Stirring was effected by an overhead stirrer. Stirring was maintained at a constant rate throughout the investigation and provided sufficient agitation to preclude the sedimentation of insoluble material during each evaluation.

The pH changes were recorded with respect to time utilizing a Beckman zeromatic pH meter with an accuracy of ±0.05 pH unit. Readings were taken at regular intervals until the pH remained constant for a period of 15 minutes. This procedure, although not indicative of *in vivo* conditions, nevertheless presents data on neutralization rates of the antacids and gives a clear picture of the speed of neutralization and maximum pH achieved.

B. Buffering Capacity.—Each commercially avail-

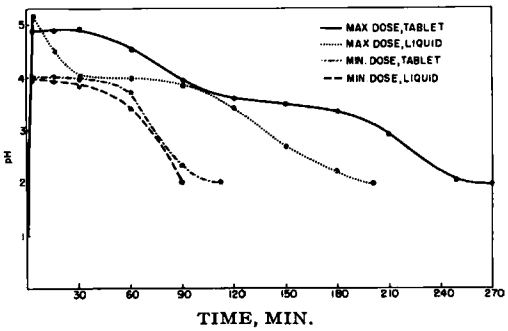

Fig. 2.—Differences in buffering capacities of minimum and maximum equivalent doses of two dosage forms of a commercial antacid containing aluminum and magnesium hydroxide, "D."

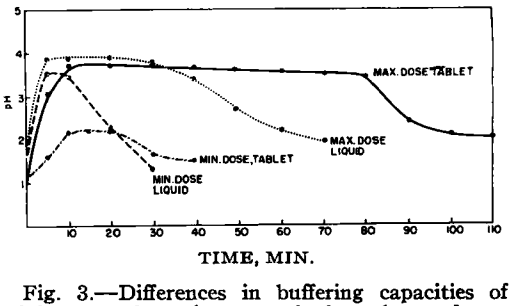

Fig. 3.—Differences in buffering capacities of minimum and maximum equivalent doses of two dosage forms of a commercial antacid containing aluminum hydroxide, "F."

TABLE IV.—BUFFERING CAPACITIES OF COMMERCIAL ANTACIDS

Code Designation	Min. Dose: Max. pH	Min. Dose: ml. 0.1 *N* HCl Neutralized	Max. Dose: Max. pH	Max. Dose: ml. 0.1 *N* HCl Neutralized
A	6.4	290	7.6	530
B	5.3	930	...	...
C	8.0	330	8.3	690
D	4.0	310	4.9	580
D-L	4.0	250	5.1	450
E	4.0	190	5.8	390
E-L	4.3	310	4.6	610
F	2.3	130	3.8	270
F-L	3.8	110	4.0	190
G	3.4	130	4.0	280
G-L	3.9	170	4.0	320
H	2.2	160	6.3	290
H-L	2.8	100	4.7	410
I	8.9	250	8.9	490
I-L	8.9	170	8.9	510
J	4.2	130	7.3	230
J-L	6.6	210	...	...
K	7.8	250	7.7	390
L	8.9	250	...	...
M	3.6	90	3.7	150
N	1.8	80	7.6	280
O	2.3	120	5.4	510
P	2.5	120	6.2	220

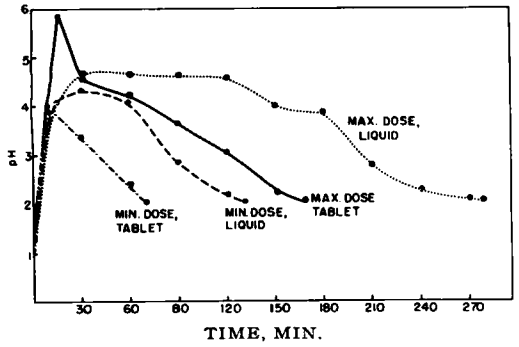

Fig. 4.—Differences in buffering capacities of minimum and maximum equivalent doses of two dosage forms of a commercial antacid containing magnesium and aluminum hydroxide, "E."

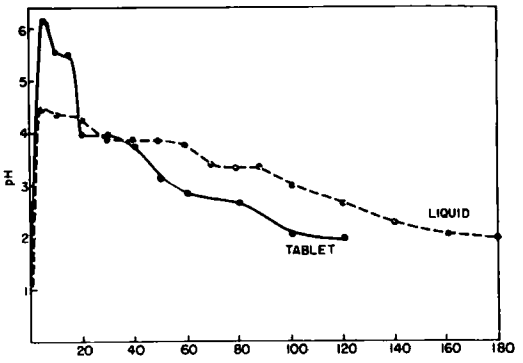

Fig. 5.—Difference in buffering capacity of the maximum equivalent doses of two dosage forms of a commercial antacid containing magnesium and aluminum hydroxide, "H."

able antacid product was evaluated as described in *Procedure A* by adding either the minimum or the maximum dose recommended by the manufacturer. Immediately after adding the dose, additional 0.1 *N* HCl was added to the reaction mixture at the rate of 2 ml. per minute until the pH returned to a base value of 2. This procedure indicated the total amount of acid consumed by the antacid under consideration as well as the time-pH relationship. An additional aspect of this phase of the study was the evaluation of the individual component chemicals currently utilized in antacid preparations.

C. Effect of Test Fluid Composition on Antacid Activity.—Commercially available antacids and antacid component chemicals were evaluated as described in *Procedure B* except that the test fluid used was modified as follows:

Test Solution 1.—Pepsin 0.32% and sodium chloride 0.2% dissolved in 0.1 *N* HCl was used. This is U.S.P. XVI artificial gastric fluid.

Test Solution 2.—Pepsin 0.32%, sodium chloride 0.2%, and peptone[1] 0.15% dissolved in 0.1 *N* HCl was used. The concentration of peptone approximates the amount of this material found in human gastric fluid (5).

These solutions were used in additional modified procedures which were designed to study the effect of polypeptides on rate of neutralization. The method outlined in *Procedure A* was employed but *Test Solution 2* was used instead of 0.1 *N* HCl.

[1] Bacto-Peptone, Difco Laboratories, Detroit, Mich.

RESULTS AND DISCUSSION

The onset of action of an antacid is a critical feature of its efficiency. This action is demonstrated by the rate of neutralization manifested by the product. The results listed in Table III indicate that in almost all cases antacid effect is observed within 5 minutes. For comparison, the time required to reach a level of pH 4 for each antacid is reported. It is evident from the data presented in Table III that there is little difference in onset of action between the liquid form of an antacid product compared to a chewable tablet.

TABLE V.—BUFFERING CAPACITIES OF INDIVIDUAL ANTACID COMPONENTS

Code Designation	Max. pH Attained	0.1 *N* HCl Neutralized, ml.
Q	8.2	130
R	4.1	110
S	3.8	330
S-L	3.8	310
T	5.9	110
T-L	5.8	100
U	7.6	230
U-L	7.4	210
V	9.6	...
W	8.5	210

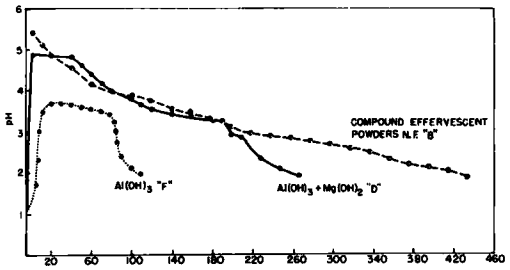

Fig. 6.—Buffering capacities of representatives of three different types of antacid preparations.

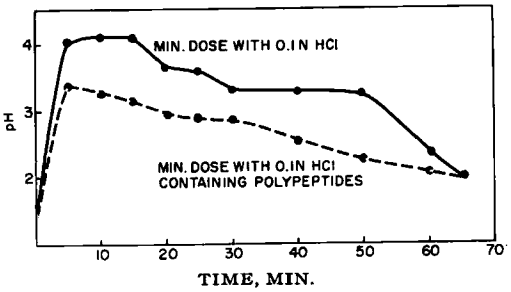

Fig. 7.—Polypeptide inhibition on buffering activity of a commercial antacid tablet containing aluminum and magnesium hydroxide, "E."

Moreover, the liquid forms of products D and E seem to be less basic than the equivalent amounts of the same components in tablet form. Figure 1 indicates that in both products D and E a lower maximum pH is attained with the liquid form. This may be attributable to the nonactive components of the suspension or the tablet which may combine with the antacid material to yield a compound of lower alkalinity upon neutralization or which may physically impede the availability of the active components. Viscosity increasing agents used to stabilize suspensions or binding agents used in tablets may possibly demonstrate this effect. This phenomenon is more clearly observed in Figs. 2 and 3 which indicate buffering capacity of antacids D and F. In each case the tablets consume a significantly greater quantity of acid compared to the liquid form.

In Fig. 3, even though the minimum dose of the liquid preparation is a superior antacid (as indicated by the area under the curve to a base line at pH 2), the equivalent dose in tablet form neutralizes

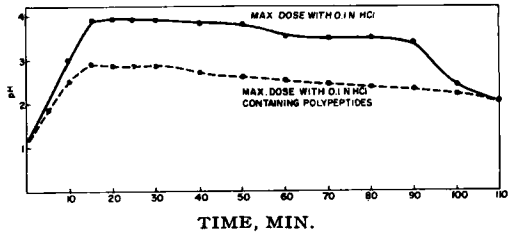

Fig. 8.—Polypeptide inhibition on buffering activity of a commercial antacid tablet containing aluminum hydroxide, "F."

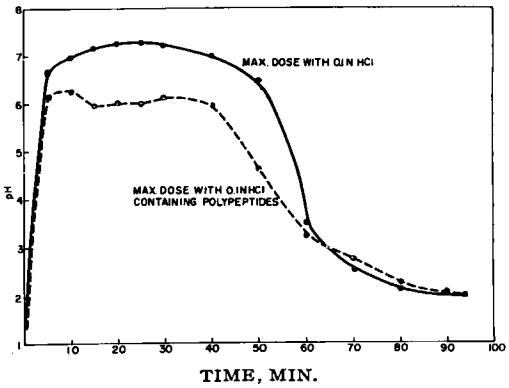

Fig. 9.—Polypeptide inhibition on buffering activity of a commercial antacid tablet containing calcium carbonate and glycine, "J."

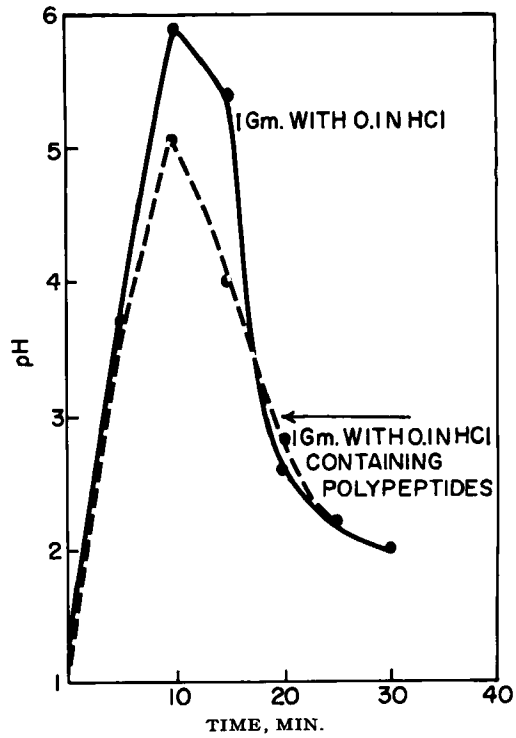

Fig. 10.—Polypeptide inhibition on buffering activity of magnesium trisilicate.

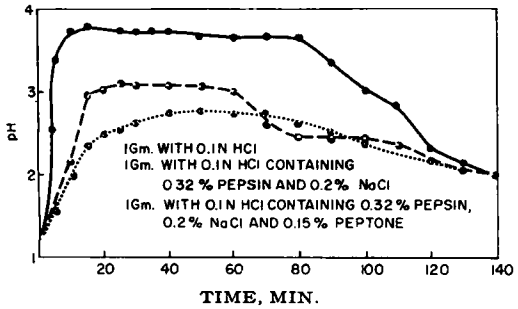

Fig. 11.—Polypeptide inhibition on buffering activity of dried aluminum hydroxide gel U.S.P. The legends identify the respective curves beginning at the top.

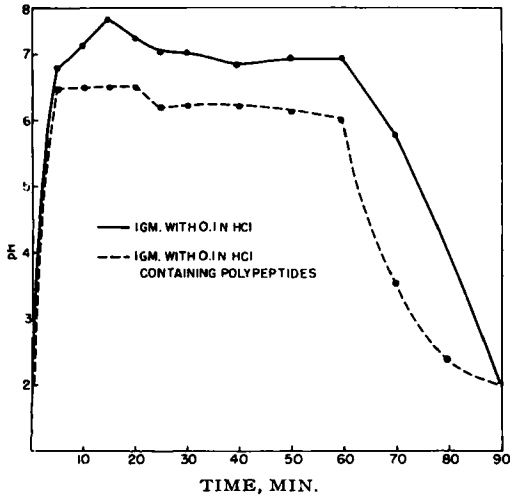

Fig. 12.—Polypeptide inhibition on buffering activity of calcium carbonate.

a greater amount of acid. If the assumption is made that a similar picture exists *in vivo*, then it is conceivable that the tablet form would be severely limited in treatment of peptic ulcer where inactivation of gastric pepsin activity due to elevated pH is desired. In such cases, the liquid form would manifest a greater degree of therapeutic activity.

On the other hand, when products E and H and their liquid equivalents, E-L and H-L, were evaluated under identical conditions, quite the opposite effects were noted. These results are depicted in Figs. 4 and 5 and indicate that the liquid showed a considerably greater neutralizing capacity compared to the equivalent dose in tablet form.

It is significant that products D and F contain aluminum hydroxide as the predominent component, while product E contains equivalent amounts of magnesium and aluminum hydroxides and product H contains magnesium trisilicate in a ratio of 2–1 to aluminum hydroxide. Auxiliary investigations are currently underway to discover whether these divergent results are due to individual product properties or the antacid components generally.

A comparison of the different types of products revealed that all the effervescent antacids tested possess a much greater buffering capacity than the noneffervescent antacids. These results are contained in Tables IV and V. Figure 6 indicates that compound effervescent powder N.F. (product B) neutralized almost twice the volume of 0.1 *N* HCl as product D. Product D is the most efficient noneffervescent compound with respect to neutralizing capacity. Of greater significance is that effervescent product B neutralizes almost four times the amount of 0.1 *N* HCl as does product F which contains aluminum hydroxide as the sole active component.

Effect of Polypeptides.—It has been demonstrated (16, 17) that polypeptides such as would be found in the stomach contents following ingestion of a meal have a decided inhibitory effect on antacid activity of aluminum hydroxide. To ascertain whether polypeptides such as pepsin and peptone exhibit a similar inhibitory effect on other antacid materials, the buffering capacities of a number of marketed antacid preparations of varying composition were evaluated in the presence of polypeptides. Figures 7–9 represent the results of these tests. These figures indicate that the inhibitory effect of polypeptides is not limited to aluminum hydroxide exclusively but is seen to occur with the other products tested which contain magnesium or calcium compounds.

Polypeptide inhibition was not observed in the

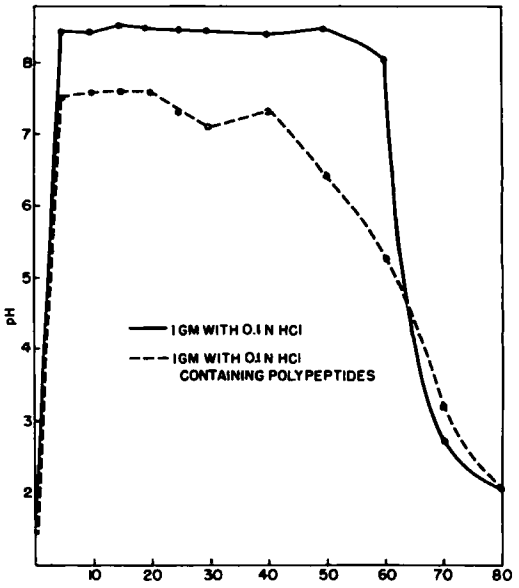

Fig. 13.—Polypeptide inhibition of buffering activity of magnesium carbonate.

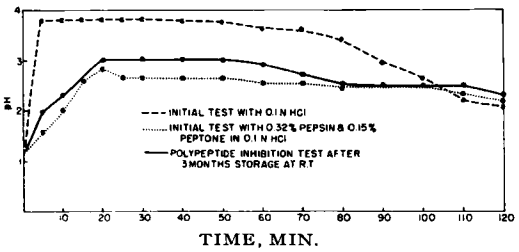

Fig. 14.—Effect of aging on polypeptide inhibition of the buffering capacity of a 10% w/v aluminum hydroxide aqueous suspension.

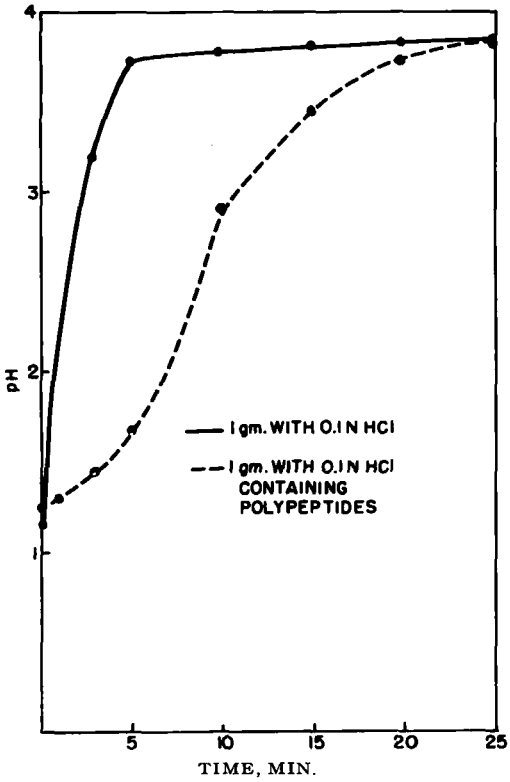

Fig. 15.—Polypeptide induced neutralization lag with dried aluminum hydroxide gel U.S.P.

liquid products tested regardless of composition. In addition, it was not demonstrated with the effervescent products tested. Increases in concentration of pepsin and peptone in the test solutions failed to cause deviations in the neutralization or buffering capacity curves of the effervescent antacids. This resistance to inhibition would seem to offer a marked advantage to the use of either the liquid form of an antacid or an effervescent antacid.

To complete the study of the inhibitory effect, a number of powdered antacid chemicals were selected and tested for buffering capacity in the presence of pepsin and peptones. Figures 10–13 demonstrate the pronounced depression in the curves for aluminum, magnesium, and calcium compounds.

Although there is a marked depression in the pH attained by an antacid in the presence of polypeptides, an important observation which has not been reported in the literature to date is that the total volume of HCl neutralized remains the same. In all cases the volume of hydrochloric acid required to return the pH to a level of 2 was the same regardless of whether pepsin and peptone were present. This indicates that the inhibition will be of little significance when considering maximum doses of calcium and magnesium compounds. Since both compounds elevate the pH well above 5 at these dosage levels, a slight decrease of 1 to 1.5 pH units will have little effect on their activity, considering that the same amount of hydrochloric acid is neutralized.

The inhibition effect, however, does become significant when considering the lower doses of these materials as well as dry aluminum compounds. It is therefore plausible to consider the possibility that the *in vivo* activity of these antacids in the presence of polypeptides will be markedly reduced or limited.

Mechanism of Inhibitory Effect.—Figure 14 depicts the results obtained when a 10% dispersion of dried aluminum hydroxide gel in distilled water was allowed to hydrate for a period of 3 months. The aging period served to decrease the polypeptide effect; these results are in agreement with those obtained with commercial liquid antacid products.

The neutralization lag was studied by subjecting 1 Gm. of dried aluminum hydroxide gel to *Test Solution 2*. Figure 15 indicates that the maximum pH level of the lower curve is the same as the upper curve. This fact together with those observed in all buffer capacity experiments, which showed the same amount of HCl neutralized with or without polypeptides, indicates that a kinetic involvement underlies these phenomena rather than some irreversible complex formation. Additional work is being conducted which will lead to a more complete elucidation of the mechanism involved.

SUMMARY

1. A comparative *in vitro* evaluation was conducted on effervescent and noneffervescent products and on liquid and solid dosage forms of the same product. It was demonstrated that the effervescent antacids were superior in buffering capacity to the noneffervescent types. Differences in efficiency were observed between solid and liquid dosage forms on an equivalent dosage basis.

2. The role of polypeptide inhibition in *in vitro* procedures was studied and produced data which indicate that a positive inhibitory effect may be observed with all solid noneffervescent antacids but not with liquid or effervescent forms.

3. The pronounced effect of polypeptides on many antacid preparations illustrates the vital role they exercise in *in vitro* evaluations and the necessity for their inclusion in all artificial test fluids.

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Tachyphylaxis III

Ephedrine

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Tachyphylaxis to ephedrine was studied in anesthetized cats by the following parameters: pressor responses (height and duration), heart rate, nictitating membrane contractions, and tone. *Levo* or *dl*-norepinephrine infusions, in contrast to epinephrine or phenylephrine infusions, given after the initial development of tachyphylaxis, produced a partial return of responses to ephedrine. Ephedrine tachyphylaxis and its specific reversal by norepinephrine are discussed.

OUR STUDIES on the reversal of neuro-sympathomimetic amine tachyphylaxis (1, 2) were continued with *l*-ephedrine sulfate, which is a well known tachyphylactogenic agent (3, 4). Its action has been postulated to be of a mixed nature (5–7), *i.e.*, it appears to act directly on smooth muscle as well as indirectly by release of norepinephrine from endogenous stores. Thus, it was of particular interest to ascertain whether the replacement of the partially depleted neurohumor, *l*-norepinephrine, would alter the development of tachyphylaxis to this sympathomimetic amine of dual action.

METHODS

l-Ephedrine sulfate was injected intravenously once every hour at a dose of 1.5 mg./Kg. or 0.75 mg./Kg. *l*-Norepinephrine bitartrate monohydrate (*l*-NOR) or *dl*-norepinephrine hydrochloride (*dl*-NOR) were dissolved in physiological salt solution to a concentration of 60 mcg./ml. and administered intravenously by continuous infusion at the rate of 2.3 mcg./minute, regardless of the weight of the animal. The infusions started 15 minutes after the second, third, or fourth ephedrine injection when tachyphylaxis was developed. In view of the differences in molecular weights between the two norepinephrine salts and of the well known lesser potency of *d*-norepinephrine, this infusion rate was arbitrarily chosen.

To ascertain the specificity of norepinephrine, phenylephrine hydrochloride (diluted to 300 mcg./ml. with physiological salt solution) was infused starting 15 minutes after the second hourly ephedrine injection at a rate of 11.6 mcg./minute, as was *l*-epinephrine hydrochloride at a rate of 4.6 mcg./minute. Dichloroisoproterenol hydrochloride (DCI), the β-receptor blocking agent (9), was administered intravenously at a dose of 3 mg./Kg. 15 minutes before the first hourly injection of ephedrine to determine the influence of β-receptors (8).

Male cats, weighing 3–4 Kg., were anesthetized with α-chloralose (80.0 mg./Kg. intraperitoneally) and pretreated with atropine sulfate (2.0 mg./Kg. intravenously). Polygalacturonic acid glycoside (PAG) was employed as an anticoagulant. The parameters measured and reported in this paper were (*a*) mean arterial blood pressure, (*b*) heart rate, (*c*) tonus, and (*d*) contractions of the nictitating membrane. Blood pressure from the left carotid artery was recorded by a Sanborn transducer (No. 267-B) on a four channel Sanborn polygraph. The changes in blood pressure responses are reported in mm. Hg and their duration in minutes. The contractions and tone of the right nictitating membrane in animals were recorded with a Grass transducer (No. FTO3) with the crystalline lens removed. The weight on the nictitating membrane was 3.2 Gm. The attenuator (Carrier preamplifier, Sanborn No. 150-1100AS) was set at $\times 5$; the responses of the nictitating membrane are reported in millimeters and read directly from the paper. Changes in heart rate were counted from the blood pressure recording with the paper speed set at 5 mm./second. The right femoral vein was cannulated for single injections and the left femoral vein for continuous drug infusions. Tracheotomy was routinely performed in all animals, although it was not necessary to use artificial respiration in any of the animals.

The first ephedrine injection was given 15 minutes after completion of surgery and pretreatment with atropine and PAG.

The data were evaluated statistically and the control animals were compared with the infused cats by the "t" test for paired experiments (10).

MATERIALS

The following drugs were used in these experiments: *l*-ephedrine sulfate,[1] *l*-norpinephrine bitartrate monohydrate,[2] *dl*-norepinephrine hydrochloride,[3] phenylephrine hydrochloride,[4] *l*-epinephrine hydrochloride,[5] dichloroisoproterenol hydrochloride,[6] α-chloralose,[7] atropine sulfate,[8] and polygalacturonic acid glycoside.[9] All doses are expressed as the weight of the respective salts.

RESULTS

Ephedrine Sulfate, 0.75 mg./Kg.

For the following reported experiments, only

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[1] New York Quinine Co., New York, N. Y.
[2] Biochemicals Corp., Cleveland, Ohio.
[3] Sigma Chemical Corp., St. Louis, Mo.
[4] Winthrop Laboratories, New York, N. Y.
[5] Marketed as Adrenalin by Parke, Davis and Co., Detroit, Mich.
[6] Eli Lilly and Co., Indianapolis, Ind.
[7] Kuhlmann Products, Paris, France.
[8] New York Quinine Co., New York, N. Y.
[9] Marketed as Mepesulfate by Hoffmann-LaRoche, Nutley, N. J.

TABLE I.—DIFFERENCES IN RESPONSES TO HOURLY INTRAVENOUS INJECTIONS OF 0.75 MG./KG. OF EPHEDRINE WITH [a] AND WITHOUT [b] CONTINUOUS INTRAVENOUS INFUSION OF *l*-NOREPINEPHRINE (*l*-NOR)

Differences in responses	Treatment	$\bar{d} \pm s\bar{d}$, 3rd to 4th	*P*, 3,4	$\bar{d} \pm s\bar{d}$, 3rd to 5th	*P*, 3,5	$\bar{d} \pm s\bar{d}$, 3rd to 6th	*P*, 3,6	$\bar{d} \pm s\bar{d}$, 3rd to 7th	*P*, 3,7
Blood pressure, mm. Hg	Without *l*-NOR	-6 ± 2.2	<0.05[c]	-6 ± 3	<0.2	-12 ± 2.9	<0.02[c]	-16 ± 0.7	<0.001[c]
	With *l*-NOR	-3.7 ± 4.6	<0.5	-9.3 ± 5.5	<0.2	-13.1 ± 6.4	<0.2	-8.1 ± 6.2	<0.3
Heart rate, beats/min.	Without *l*-NOR	-9.6 ± 2.4	<0.02[c]	-12.4 ± 3.5	<0.05[c]	-14.4 ± 2.4	<0.01[c]	-14.4 ± 2.4	<0.001[c]
	With *l*-NOR	$+3 \pm 3.4$	<0.5	$+3 \pm 6$	<0.7	-9 ± 3	<0.1	-9 ± 3	<0.1

[a] Number of cats, 4; weight, 3.0 to 3.5 Kg. [b] Number of cats, 5; weight 3.0 to 3.5 Kg. [c] Indicates statistical significance.

those cats were chosen which showed clear-cut tachyphylaxis after the third hourly injection. This group of animals was comprised of 13 cats, five of which were used as controls and eight of which were infused with norepinephrine.

A dose of 0.75 mg./Kg. of ephedrine caused invariably reduced pressor responses to the second hourly injection when compared with the first, but in many animals the responses thereafter showed very little (if any) additional decrease. In some cats this dose of ephedrine caused continued tachyphylaxis on blood pressure as could be judged from the substantial diminution of the pressor response to the injections thereafter. The duration of the responses also decreased with the repeated injections of ephedrine, from an average of 6 minutes to an average of 2 minutes.

Infusion With *l*-Norepinephrine (*l*-NOR).—With the infusion rate of *l*-NOR employed, the initial mean arterial blood pressure was little changed but decreased slightly toward the end of the experiments.

The duration of the blood pressure responses to ephedrine was not altered by the infusion of *l*-NOR when compared with the controls.

In Table I, the differences in the blood pressure responses and changes in heart rate from the fourth through seventh hourly injections of ephedrine in the absence and presence of *l*-NOR are compared with the third hourly responses. Five cats received only hourly ephedrine injections (controls), whereas four cats were continuously infused intravenously with *l*-NOR starting after the third hourly injection of ephedrine.

As can be seen from Table I, the decrease in the blood pressure responses to ephedrine in the control animals became progressively more significant with time, except for the fifth hour. The differences between the responses in the *l*-NOR-treated animals were not significant throughout this period of time, showing that *l*-norepinephrine prevented the further development of ephedrine tachyphylaxis. Also the heart-rate changes in the control animals were significantly reduced and *l*-NOR infusion counteracted this reduction.

The nictitating membrane contractions induced by ephedrine did not show such a clear-cut effect of *l*-NOR. In all of the control animals the contractions were progressively and significantly reduced. When compared with the third hourly response, *l*-NOR showed its reversing effect only at the fourth and sixth hours. In the control animals the nictitating membrane tone increased significantly in the fourth and fifth hours and thereafter remained essentially the same. In the *l*-NOR treated animals, the nictitating membrane tone plateaued after the fourth hourly injection of ephedrine.

Infusion with *dl*-Norepinephrine (*dl*-NOR).—The infusion of *dl*-NOR in doses already referred to did not alter the initial mean arterial blood pressure significantly. The duration of the blood pressure responses to ephedrine was essentially unaltered by the infusion of *dl*-NOR.

The effect of infusion of *dl*-NOR on the changes in blood pressure response, the changes in heart rate, and the nictitating membrane tone to ephedrine was identical with that described previously for *l*-NOR. The effect of *dl*-NOR on the nictitating membrane contractions to ephedrine is summarized in Table II.

As can be clearly seen, the nictitating membrane contractions to ephedrine show significant reductions upon the progressive hourly administration of ephedrine in the control cats and *dl*-NOR prevents this reduction. This shows that *dl*-NOR, in contrast to *l*-NOR, is capable of counteracting ephedrine tachyphylaxis on this organ.

Ephedrine Sulfate, 1.5 mg./Kg.

A dose of 1.5 mg./Kg. of ephedrine caused significant reductions of the mean arterial blood pressure and heart-rate responses upon repeated hourly injections in all control animals.

TABLE II.—DIFFERENCES OF NICTITATING MEMBRANE CONTRACTIONS (MM.) TO 0.75 MG./KG. OF EPHEDRINE INJECTED INTRAVENOUSLY ONCE EVERY HOUR

Treatment	$\bar{d} \pm s\bar{d}$, 3rd to 4th	*P*, 3,4	$\bar{d} \pm s\bar{d}$, 3rd to 5th	*P*, 3,5	$\bar{d} \pm s\bar{d}$, 3rd to 6th	*P*, 3,6	$\bar{d} \pm s\bar{d}$, 3rd to 7th	*P*, 3,7
Without *dl*-NOR[a]	-2.4 ± 0.48	<0.01[c]	-3.7 ± 0.7	<0.01[c]	-4.6 ± 1.3	<0.05[c]	-5.1 ± 1.3	<0.02[c]
With *dl*-NOR[b,d]	$+1.5 \pm 1.2$	<0.3	$+0.9 \pm 1.5$	<0.6	$+0.5 \pm 1.2$	<0.7	-0.8 ± 0.9	<0.5

[a] Number of cats, 5; weight, 3.0 to 4.5 Kg. [b] Number of cats, 4; weight, 3.0 to 4.0 Kg. [c] Indicates statistical significance. [d] *dl*-Norepinephrine (*dl*-NOR) was continuously infused intravenously starting 15 minutes after the third hourly and ending 30 minutes after the seventh hourly ephedrine injection at the rate of 2.3 mcg./min.

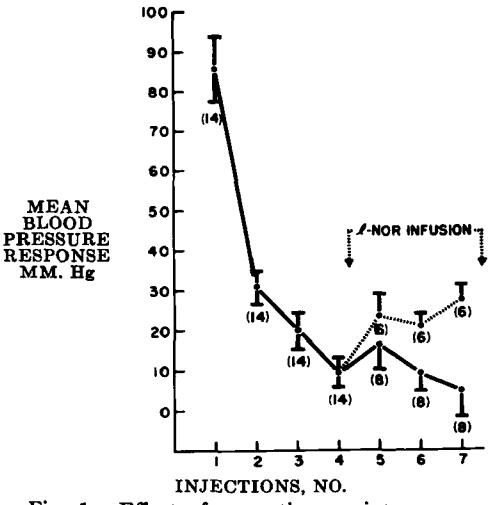

Fig. 1.—Effect of a continuous intravenous infusion of *l*-norepinephrine (*l*-NOR, 2.3 mcg./minute) upon the mean arterial blood pressure responses to ephedrine (1.5 mg./Kg.) injected intravenously once every hour. The *l*-norepinephrine infusion was started 15 minutes after the fourth and ended 30 minutes following the seventh hourly ephedrine injection in six of 14 cats. The remaining eight animals were given only the hourly injections of ephedrine (controls). The pressor responses to ephedrine of the control and *l*-NOR-infused groups of cats were pooled for the first four hourly injections.

With this dose of ephedrine the initial mean arterial blood pressure increased slightly during the course of the experiments in all control cats (average, +28 mm. Hg).

The duration of the blood pressure responses to this dose of ephedrine showed a greater reduction than that observed, following the repeated hourly administration of the lower dose (0.75 mg./Kg.), *i.e.*, from an average of 8 minutes to an average of less than 1 minute.

Infusion with *l*-Norepinephrine (*l*-NOR).—The rate of infusion of *l*-norepinephrine, 2.3 mcg./minute, was determined in pilot studies and was used in these experiments. When infusion rates of *l*-NOR *smaller* than 2.3 mcg./minute were employed in six cats, complete tachyphylaxis to the pressor effects of ephedrine developed. When a *higher* infusion rate of *l*-NOR was used in six cats, the initial pressor responses to ephedrine following *l*-norepinephrine infusions were considerably augmented. However, *l*-norepinephrine at these *higher* infusion rates did not prevent the terminal development of tachyphylaxis. *l*-NOR infusion at the rate of 2.3 mcg./minute increased the initial mean arterial blood pressure by an average of +25 mm. Hg, which remained essentially constant throughout the experiments.

The duration of the blood pressure responses to ephedrine in the presence of *l*-NOR was less reduced than in the control animals (from an average of 8 minutes to an average of 2 minutes).

Figure 1 summarizes the pooled mean arterial blood pressure changes for all 14 animals regarding the first four hourly injections of ephedrine. Eight of these 14 animals served as the ephedrine controls. In the remaining six animals *l*-NOR infusion (2.3 mcg./minute) was started 15 minutes after the fourth injection and continued to the end of the experiment. It can be clearly seen that the infusion *l*-NOR *at this rate* will reverse tachyphylaxis to the level of the second hourly injection of ephedrine but not fully. Thus, the reversal of tachyphylaxis in this experiment is partial but not complete. The "protective" effect of *l*-NOR on the pressor responses to ephedrine (fifth to the seventh hour) is statistically significant (P = <0.02 to <0.01). In summary, we may say that an infusion rate of *l*-NOR of 2.3 mcg./minute was effective in reversing the development of tachyphylaxis of the ephedrine pressor responses, but that infusion rates *higher* or *lower* than 2.3 mcg./minute were not effective.

The data for the changes of heart rate in these same animals are summarized in Fig. 2. The changes in heart rate to ephedrine become progressively less reduced to repeated injections of the amine after the second hour.

Between the first and second injections of ephedrine the heart rate is elevated and remains rapid throughout the experiment.

The continuous infusion of *l*-NOR partially reverses the ever decreasing heart-rate response to ephedrine, although this reversal is statistically significant only at the seventh injection of ephedrine (P = <0.02).

A summary of the nictitating membrane tone and contractions of these same animals is given in Table III.

The nictitating membrane responses to ephedrine of all 14 animals were pooled for the first 4 hours, and the responses of the eight control animals and the six *l*-NOR-infused animals are shown separately for the fifth through the seventh hour. As can be seen, the sums of the nictitating membrane contraction and tone are nearly constant throughout the experimental period, both in the presence and absence of *l*-NOR.

From the fifth to the seventh hour the eight control cats show a progressive decrease of the nictitat-

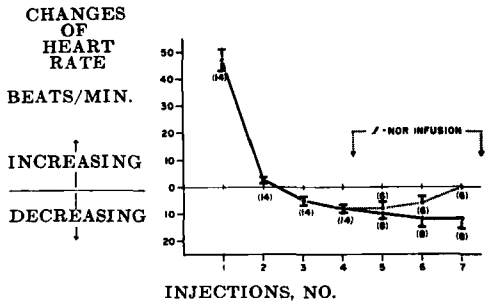

Fig. 2.—Effect of a continuous intravenous infusion of *l*-norepinephrine (*l*-NOR, 2.3 mcg./minute) upon the ephedrine-induced (1.5 mg./Kg. intravenously) mean changes in heart rate. The ephedrine was injected once every hour. The *l*-norepinephrine infusion was started 15 minutes after the fourth and ended 30 minutes following the seventh hourly ephedrine injection in six of 14 cats. The remaining eight animals were given only the hourly injections of ephedrine (controls). The ephedrine-induced changes in heart rate observed in the control and *l*-NOR-infused groups of cats were pooled for the first four hourly injections.

TABLE III.—NICTITATING MEMBRANE RESPONSES (MM.) TO 1.5 MG./KG. OF EPHEDRINE INJECTED INTRAVENOUSLY ONCE EVERY HOUR[a]

Injection, No. →	1	2	3	4	5	6	7
Without *l*-NOR (No. cats, 8)							
1. Contraction	14.8 ± 0.8	7.0 ± 0.6	4.8 ± 0.4	3.8 ± 0.4	4.8 ± 0.4	3.8 ± 0.6	3.2 ± 0.8
2. Tone	7.8 ± 0.06	17.6 ± 0.6	19.4 ± 0.8	19.0 ± 0.6	17.6 ± 0.8	18.0 ± 1.2	18.6 ± 1.4
3. Sum of 1 + 2	22.4	24.6	24.2	22.8	22.4	21.8	21.8
With *l*-NOR[b] (No. cats, 6)							
4. Contraction	...	...	...	...	2.6 ± 0.4	2.6 ± 0.4	3.0 ± 0.4
5. Tone	...	...	...	...	20.2 ± 0.6	20.4 ± 1.2	19.6 ± 1.2
6. Sum of 4 + 5	...	...	...	...	22.8	23.0	22.6

[a] The responses of all 14 cats were pooled for the first four hourly ephedrine injections. [b] *l*-Norepinephrine (*l*-NOR) was continuously infused intravenously starting 15 minutes after the fourth and ending 30 minutes after the seventh hourly ephedrine injection at the rate of 2.3 mcg./min.

ing membrane contractions and a simultaneous increase in tone. The nictitating membrane tone of the *l*-NOR-treated cats is higher than that of the control animals, and the contractions are lower. Nevertheless, *l*-NOR might exert a slight "protective" effect since a further decrease in contractions from the fifth to the seventh hour is absent. The "protective" effect of *l*-NOR on these nictitating membrane contractions to ephedrine cannot be demonstrated by statistical methods.

We questioned whether repeated hourly injections of 1.5 mg./Kg. of ephedrine would depress the responses to single doses of *l*-NOR.

Ephedrine tachyphylaxis was permitted to develop through the fourth injection. Single repeated (three times) injections of a geometric series of *l*-NOR (0.25 to 1.0 mcg./Kg.) were administered intravenously before the first and after the fourth ephedrine injections. The pressor responses to the single injections of *l*-NOR were biphasic and ranged *initially* from +77 to −3 mm. Hg to the low dose, to +103 to −19 mm. Hg to the high dose of *l*-NOR. After the fourth ephedrine injection, the responses to the low dsse of *l*-NOR were +73 to −12 mm. Hg and to the high dose +86 to −9 mm. Hg. Thus, in the presence of ephedrine tachyphylaxis only insignificant reductions of the pressor responses to the single injections of *l*-NOR could be observed.

Infusion with *dl*-Norepinephrine (*dl*-NOR).—In addition to *l*-NOR, *dl*-NOR was also employed to study its effect on the development of ephedrine tachyphylaxis. The infusion of *dl*-NOR essentially failed to alter the initial mean arterial blood pressure at the rates administered.

dl-NOR was infused (2.3 mcg./minute) starting 15 minutes after the fourth hourly injection of ephedrine, and this catecholamine infusion was continued to the end of each experiment. It is obvious that *dl*-NOR is less potent than *l*-NOR; nevertheless, the results observed on the pressor responses to ephedrine are similar, though the heart rate responses appear to be less marked. Furthermore, *dl*-NOR caused only an insignificant increase in the duration of the blood pressure responses to ephedrine when compared with control animals. (Average first response, 10 minutes; average seventh response, 1.1 minutes.)

It may be observed in Table IV that in cats infused with *dl*-NOR the ephedrine-induced contractions of the nictitating membrane are significantly higher than those of the control animals. The increase in nictitating membrane tone is considerably less pronounced in the animals infused with *dl*-NOR compared with that observed in the cats infused with *l*-NOR.

Infusion with Other Sympathomimetic Amines.—To ascertain the specificity of norepinephrine in partially counteracting ephedrine tachyphylaxis, five cats were infused with *l*-epinephrine and three cats with phenylephrine (see *Materials* and *Methods*).

TABLE IV.—DIFFERENCES IN RESPONSES TO HOURLY INTRAVENOUS INJECTIONS OF 1.5 MG./KG. OF EPHEDRINE WITH[a] AND WITHOUT[b] CONTINUOUS INTRAVENOUS INFUSION OF *dl*-NOREPINEPHRINE (*dl*-NOR) STARTING 15 MINUTES AFTER THE FOURTH HOURLY EPHEDRINE INJECTION

Differences in responses	Treatment	$\bar{d} \pm \overline{sd}$, 4th to 5th	*P*, 4,5	$\bar{d} \pm \overline{sd}$, 4th to 6th	*P*, 4,6	$\bar{d} \pm \overline{sd}$, 4th to 7th	*P*, 4,7
Blood pressure, mm. Hg	Without *dl*-NOR	− 3.8 ± 3.0	<0.3	− 9.2 ± 4.9	<0.2	−15.0 ± 3.0	<0.01[c]
	With *dl*-NOR	+18.8 ± 5.6	<0.05[c]	+18.3 ± 3.8	<0.01[c]	+23.6 ± 4.8	<0.01[c]
Heart rate, beats/min.	Without *dl*-NOR	− 2.0 ± 2.0	<0.4	− 4.0 ± 1.8	<0.1	− 4.8 ± 2.9	<0.2
	With *dl*-NOR	− 2.0 ± 2.0	<0.4	− 2.0 ± 2.0	<0.4	− 2.4 ± 2.4	<0.4
Nictitating membrane contraction, mm.	Without *dl*-NOR	− 0.3 ± 0.2	<0.2	− 1.3 ± 0.4	<0.02[c]	− 2.2 ± 0.4	<0.01[c]
	With *dl*-NOR	− 0.2 ± 0.2	<0.4	− 0.6 ± 0.3	<0.1	−0.7 ± 0.4	<0.2
Nictitating membrane tone, mm.	Without *dl*-NOR	−0.03 ± 0.8	<1.0	+ 0.2 ± 0.5	<0.8	+ 0.8 ± 0.5	<0.2
	With *dl*-NOR	+ 0.1 ± 0.3	<0.7	+ 0.2 ± 0.5	<0.8	± 0.01 ± 0.7	<1.0

[a] Number of cats, 6; weight 3.0 to 4.5 Kg.; infusion rate of *dl*-NOR 2.3 mcg./min. [b] Number of cats, 8; weight 3.0 to 4.5 Kg. [c] Indicates statistical significance.

None of the four parameters measured was influenced by the infusion of these two sympathomimetic amines when compared with the controls.

Effect of Dichloroisoproterenol (DCI) upon Development of Ephedrine Tachyphylaxis

Since DCI was shown to antagonize the effects of ephedrine on the heart (11), it was of interest to test the influence of this β-receptor blocking agent on ephedrine tachyphylaxis and its reversal by norepinephrine. These experiments were carried out for the duration of six hourly ephedrine (1.5 mg./Kg.) injections, and DCI (3 mg./Kg.) was administered intravenously 15 minutes before the first ephedrine injection. Four cats were tested with DCI and ephedrine alone. In contrast to the cats not pretreated with DCI, which showed a slight increase in the initial mean arterial blood pressure throughout the experiments, these DCI-treated cats showed a slight decrease (average, −29 mm. Hg). No change in heart-rate response to ephedrine was observed throughout the experimental period, although the duration of action of DCI at this dose was reported to be considerably shorter than 6 hours (11). In two cats the initial heart rate remained unchanged; however, in the other two animals the initial heart rate increased somewhat after the third hour without further increase following the fourth, fifth, and sixth ephedrine injections. Nevertheless, tachyphylaxis development to ephedrine-induced blood pressure responses, nictitating membrane contractions, and the increase in nictitating membrane tone paralleled the effects of ephedrine in animals without DCI administration. However, the duration of the blood pressure responses to ephedrine was considerably prolonged (average of 40 minutes to the first injection and an average of 25 minutes to the sixth ephedrine injection). It should be emphasized that the considerable variations of the pressor effects to the first injection of ephedrine were largely eliminated in cats pretreated with DCI. This also resulted in lower pressor effects upon the first ephedrine injection.

Three other cats received DCI (3 mg./Kg.) 15 minutes before the first injection of ephedrine and were infused with *l*-NOR starting 15 minutes after the second ephedrine injection. This catecholamine was infused continuously to the end of the experiment. The initial mean arterial blood pressure in these animals was not significantly altered, and the duration of the pressor responses to ephedrine were essentially the same as those observed in the control animals. Under these experimental conditions *l*-NOR did not reverse the ephedrine-induced tachyphylaxis on the blood pressure. In these three cats the heart rate did not increase to ephedrine administration; therefore, even during the infusion of *l*-NOR, ephedrine failed to accelerate the heart. However, *l*-NOR itself produced a slight increase in the heart rate throughout the experiment.

The only parameter measured which showed statistically significant reversal of ephedrine tachyphylaxis by *l*-NOR infusion in the DCI-pretreated animals were the contractions of the nictitating membrane.

DISCUSSION

The above experiments demonstrating the tachyphylaxis of sympathomimetic amines were designed in accordance with the original method of Tainter (12).

For the present studies we arrived at a dose of ephedrine (1.5 mg./Kg.) to which tachyphylaxis of all parameters measured was observed in all experimental animals. We also employed a borderline tachyphylactic dose (0.75 mg./Kg.).

In ephedrine (1.5 or 2.5 mg./Kg.) tachyphylaxis (1), the main differences in the blood pressure responses to this drug occur between the first and second hourly injections and those to the heart rate between the first and third hourly administrations (first phase of tachyphylaxis). The reductions in these responses thereafter become progressively less pronounced (second phase of tachyphylaxis). The separation of the two phases of ephedrine tachyphylaxis becomes considerably less distinct with the 0.75 mg./Kg. dosage. There is little or no disagreement that norepinephrine release is involved in the indirect action of the sympathomimetic amines such as ephedrine. However, the direct action of these amines may be attributed to a reversible combination with α-adrenergic receptors (action abolished or greatly diminished by α-adrenergic blocking agents) and/or to a similar reversible combination with other unknown receptors in the smooth and cardiac muscle. Blockade of the cardiac β-receptors by DCI reduces the first blood pressure response to ephedrine and thereby makes the first phase of tachyphylaxis less pronounced without essentially altering the prolonged second phase.

With the infusion of *l*-NOR or *dl*-NOR we were able to overcome the second phase of tachyphylaxis. DCI, in the presence of which the first phase of tachyphylaxis is greatly reduced, prevented this reversing effect of norepinephrine on blood pressure. Thus, we must attribute a crucial role to the heart in this return of responses; however, the heart alone cannot fully account for the partial reversal by norepinephrine of the ephedrine tachyphylaxis because the contractions of the nictitating membrane were also partially re-established. Since by the infusion of norepinephrine we were unable to reverse the first phase of tachyphylaxis to ephedrine at 1.5 mg./Kg., and could not reverse either phase of tachyphylaxis to 2.5 mg./Kg., as previously reported (2), we must assume a partly irreversible direct, possibly toxic, action of ephedrine on the heart and smooth muscle, which becomes apparent immediately upon the first injection of the amine. It was observed in our laboratory that ephedrine in high concentration increased the permeability of the vessels of the rat-*meso*-appendix (13). Since ephedrine is a drug of prolonged duration of action, its toxicity or irreversible effect might also be due to such an alteration of vascular, *i.e.*, smooth muscle permeability. Another possibility is a depression by ephedrine of the secretion of adrenocorticoids which, according to Ramey and Goldstein (14), maintain the integrity and responsiveness of tissues in the process of reacting to norepinephrine.

The effects of the drugs on the smooth muscle of the nictitating membrane are difficult to evaluate unequivocally because the tone of this organ never returns to control levels after the first injection of ephedrine. Nevertheless, the contractions of the

nictitating membrane were partially re-established in the absence and presence of DCI. These results should make it obvious that we cannot expect all parameters made tachyphylactic by ephedrine to be simultaneously reversed (partially or completely) by an infusion of norepinephrine.

The observations made on blood pressure responses to single norepinephrine injections preceding and following ephedrine tachyphylaxis development exclude adrenergic contractile receptor saturation as an explanation for ephedrine tachyphylaxis. This is further corroborated by the fact that norepinephrine can be infused in amounts too large to prevent reversal which represents true "receptor saturation." This is not in complete agreement with the suggestions of Winder (15) and of Horita, West, and Dille (16) that "receptor saturation" is the true explanation for tachyphylaxis to sympathomimetic amines.

In view of the above considerations, full reversal of both phases of ephedrine tachyphylaxis with norepinephrine could not be expected. Nevertheless, our original postulate (that the loss of norepinephrine from critical sites as an important etiological factor in the development of sympathomimetic amine tachyphylaxis) was fully substantiated.

Norepinephrine shows a unique specificity since epinephrine cannot be substituted in its place as a reversing agent. The precursors of norepinephrine may be effective in reversing ephedrine tachyphylaxis if the synthesis of norepinephrine is not influenced by the presence of large concentrations of ephedrine. To evaluate fully the nature of ephedrine tachyphylaxis on smooth muscle, the nictitating membrane might constitute a very reliable test organ *in situ* if studied under more favorable conditions, such as by close-arterial injections. We agree with the findings of Trendelenburg, *et al.* (17), that ephedrine is neither a purely directly acting nor purely indirectly acting sympathomimetic amine, and this fact may be responsible for only the partial reversal of ephedrine tachyphylaxis by norepinephrine infusions.

In view of the dual nature of ephedrine, which is further substantiated by our observations, it is realized that this amine is not the best drug to demonstrate fully the phenomenon of tachyphylaxis reversal. We are investigating, therefore, tachyphylaxis reversal by norepinephrine of sympathomimetic amines reported to have a greater indirect effect than ephedrine (5–7, 17), *e.g.*, tyramine and amphetamine. These results have been reported in part and will be published in the near future.

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Studies of Preservatives of Poliomyelitis (Salk) Vaccine I

Benzethonium Chloride

By HILLIARD PIVNICK, J. M. TRACY, and D. G. GLASS

Several antimicrobial materials may be present in killed Salk poliomyelitis vaccine. They are (*a*) antibiotics which are added to the tissue culture to inhibit growth of bacterial contaminants without inhibiting growth of the poliomyelitis virus, (*b*) formaldehyde added to kill the virus, and (*c*) preservatives such as benzethonium chloride added to the finished vaccine to prevent the growth of bacterial and fungal contaminants. The stable antibiotics contribute considerable antibacterial activity; the formaldehyde, if not neutralized by bisulfite, is an effective, stable antibacterial agent with some antifungal activity. Benzethonium chloride adds little, if any, antibacterial activity to that obtained either by antibiotics or formaldehyde but does furnish some antifungal activity.

PRESERVATIVES for killed poliomyelitis (Salk) vaccine must be chosen with special care because the antigenic potency of the vaccine is easily destroyed. Benzethonium chloride (BEC) has been used as a preservative for poliomyelitis vaccine and has been found to cause no destruction of the virus antigen when used in concentrations of about 25 p.p.m. (1). This concentration

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nictitating membrane were partially re-established in the absence and presence of DCI. These results should make it obvious that we cannot expect all parameters made tachyphylactic by ephedrine to be simultaneously reversed (partially or completely) by an infusion of norepinephrine.

The observations made on blood pressure responses to single norepinephrine injections preceding and following ephedrine tachyphylaxis development exclude adrenergic contractile receptor saturation as an explanation for ephedrine tachyphylaxis. This is further corroborated by the fact that norepinephrine can be infused in amounts too large to prevent reversal which represents true "receptor saturation." This is not in complete agreement with the suggestions of Winder (15) and of Horita, West, and Dille (16) that "receptor saturation" is the true explanation for tachyphylaxis to sympathomimetic amines.

In view of the above considerations, full reversal of both phases of ephedrine tachyphylaxis with norepinephrine could not be expected. Nevertheless, our original postulate (that the loss of norepinephrine from critical sites as an important etiological factor in the development of sympathomimetic amine tachyphylaxis) was fully substantiated.

Norepinephrine shows a unique specificity since epinephrine cannot be substituted in its place as a reversing agent. The precursors of norepinephrine may be effective in reversing ephedrine tachyphylaxis if the synthesis of norepinephrine is not influenced by the presence of large concentrations of ephedrine. To evaluate fully the nature of ephedrine tachyphylaxis on smooth muscle, the nictitating membrane might constitute a very reliable test organ *in situ* if studied under more favorable conditions, such as by close-arterial injections. We agree with the findings of Trendelenburg, *et al.* (17), that ephedrine is neither a purely directly acting nor purely indirectly acting sympathomimetic amine, and this fact may be responsible for only the partial reversal of ephedrine tachyphylaxis by norepinephrine infusions.

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of BEC is a good preservative against fungi and some G-positive bacteria but has little or no effect against many G-negative species, especially members of the genus *Pseudomonas* (2–4).

Besides the preservatives which are added to poliomyelitis vaccine at the end of the manufacturing process, other antimicrobial materials are added during the manufacture of the vaccine. Antibiotics such as streptomycin, neomycin, and polymyxin B are added to the tissue culture fluid to control bacterial contaminants. Some of these antibiotics, depending on their stability, will be present in the finished vaccine. In addition, formaldehyde is added to kill the virus and, unless neutralized, about three-quarters of the original amount of this compound will still be present in the finished vaccine. Thus poliomyelitis vaccine may possess considerable preservative activity without the addition of extra preservatives.

Unfortunately, despite the numerous antimicrobial compounds, contaminants do grow in poliomyelitis vaccine.

During a 3-year period, 13 partially used multiple-dose vials of poliomyelitis vaccine or poliomyelitis vaccine combined with other antigens were returned to this laboratory because of apparent contamination. All of these vials had been preserved by the addition of 25 p.p.m. of BEC; all contained some residue of the antibiotics added to the tissue culture medium. None of them, however, contained residual formaldehyde as it had been neutralized by sodium bisulfite. Two of the vials were contaminated with molds. The remainder yielded species of the genus *Pseudomonas* and two of these contained *Pseudomonas aeruginosa*. This species may cause fatal septicemia (5, 6).

All the vials had been entered and part of the contents withdrawn. Three of them, each from a different lot of poliomyelitis vaccine, were returned by a single physician, and each yielded the same species of *Pseudomonas*, but this species was not encountered in the other contaminated vials. No unused vials have shown contamination. Obviously, the entry of nonsterile needles had introduced microorganisms which had grown despite the presence of BEC. Apparently BEC was inadequate as a preservative; this report attempts to explain the reasons for the inadequacy.

Table I.—Cultures Used

Code	Culture	Code	Culture
C-1	*Pseudomonas sp.*[a]	C-12	*Salmonella paratyphi* C[c]
C-2	*Pseudomonas sp.*[a]	C-13	*Pseudomonas aeruginosa*[b]
C-3	*Pseudomonas sp.*[a]	C-16	*Pseudomonas aeruginosa*[b]
C-4	*Pseudomonas aeruginosa*[a]	C-27	*Saccharomyces ellipsoideus*[e]
C-5	*Pseudomonas sp.*[a]	C-28	*Debaromyces kloeckeri*[e]
C-6	*Pseudomonas aeruginosa*[b]	C-29	*Saccharomyces rouxii*[e]
C-8	*Shigella flexneri*[c]	C-30	*Rhodotorula glutinis*[e]
C-9	*Proteus sp.*[b]	C-32	*Rhizopus oryzae*[e]
C-10	*Staphylococcus aureus*[d]	C-33	*Fusarium lini*[e]
C-11	*Staphylococcus aureus*[d]	C-35	*Circinella spinosa*[e]

[a] Contaminated poliomyelitis vaccine. [b] Contaminated tissue culture. [c] Sick monkey. [d] Clinical laboratory. [e] Stock cultures from Ontario Agricultural College, Guelph, Ontario, Canada.

EXPERIMENTAL

Vaccines and Other Preserved Media

During this investigation many preparations, described below, were preserved and challenged.

HB597 Medium.—This is a nutrient solution used in tissue culture for the growth of poliomyelitis virus. It differs from medium 199 of Morgan, *et al.* (7), in that it contains no purines, pyrimidines, ribose, deoxyribose, or adenosinetriphosphate. In addition, Hanks' balanced salt solution is used instead of Earle's solution.

Killed Poliomyelitis (Salk) Vaccine.—This consists of HB597 medium with antibiotics, virus of the three types of poliomyelitis, soluble residue from the monkey kidney cell tissue culture, formaldehyde which may or may not be neutralized by sodium bisulfite, and a preservative. During these experiments, as discussed later, the antibiotics, preservatives, and formaldehyde have been varied.

Diphtheria - Pertussis - Tetanus - Poliomyelitis (DPT Polio) Vaccine.—This is a mixture of diphtheria and tetanus toxoids, pertussis vaccine, and Salk vaccine. The poliomyelitis component of this mixture is 96% of the total volume.

Preservatives

BEC, at a concentration of 0.5% in Hanks'

Table II.—BEC in Commercially Produced Vaccines

Manufacturer	Vaccine Type	BEC, p.p.m.
A	Poliomyelitis[a]	18
A	DPT Polio[b]	16
B	DPT Polio[b]	12
C	Poliomyelitis (5 lots)[a]	25 ± 1
C	DPT Polio (10 lots)[b]	6 ± .05

[a] Trivalent poliomyelitis vaccine (Salk vaccine). [b] Salk vaccine with diphtheria and tetanus toxoids and pertussis vaccine.

Table III.—Recovery of BEC from Poliomyelitis Vaccine Dispensed in Different Sized Containers

BEC Added, p.p.m.	BEC Found by Analysis, p.p.m. In 10-ml. vials	In 3-ml. vials	In 1-ml. ampuls
31	24.6 (4)[a]		
28	22.4 (12)	22.1 (5)	20.5 (9)

[a] Number of lots examined indicated by parentheses.

TABLE IV.—BEC CONTENT OF FOUR LOTS OF DPT POLIO VACCINE COMPARED WITH THAT OF THE POLIOMYELITIS VACCINE USED IN MAKING THEM

Lot	Polio Vaccine, BEC Content	DPT Polio Vaccine, BEC Content
1	25	7
2	28	8
3	26	7
4	23	9

balanced salt solution, was added with constant mixing to obtain the desired concentration in the medium or vaccine. Analysis of BEC was by a modification (8) of the method of Auerbach (9). Antibiotics were determined quantitatively in the vaccine by usual methods (10). When more than one antibiotic was present, assay organisms were used which were resistant to all antibiotics except the one being measured. Formaldehyde was determined by the method of Nash (11) and by a modification of the Nash method (12).

Bacteria

All bacterial cultures (see Table I) were stored in the lyophilized state or on nutrient agar slopes at −20°. To obtain cells for challenging preserved vaccines, Difco[1] nutrient broth was inoculated and incubated 2 days at 30°. During the second day of incubation the culture was aerated on a reciprocating shaker. The broth culture was then sedimented by centrifugation and resuspended to one-fifth of the original volume in Difco tryptose (0.1%)-saline (0.5%). Counts of viable cells in these suspensions were made on numerous occasions and found to vary from 0.5 to 5.0 × 10^9 cells per ml.

Yeasts and Molds

These cultures (see Table I) were stored at 4° on Difco Sabouraud agar. To obtain material for challenging samples they were grown for 5 days at 30° on Sabouraud agar slopes, washed off with tryptose-saline, and shaken vigorously to break up clumps. Several determinations indicated viable populations of 10^7 and 10^8 cells per ml.

Challenge of Preserved Systems

Medium or vaccines were dispensed, 4 ml. per screw-capped test tube, and challenged with 0.1 ml. of the suspended culture undiluted, diluted 1 × 10^{-3} and 1 × 10^{-6}.

All tubes were incubated at room temperature (20 ± 4°) and examined after 4, 11, and 30 or 60 days for macroscopic growth and/or acid production. Tubes in some experiments were incubated at 4°. When there was doubt concerning growth, media challenged with bacteria were subcultured to Difco nutrient broth or a neutralizing broth (13), and fungi were subcultured to Sabouraud agar. If positive, the medium was considered to have macroscopic evidence of growth. Tubes which showed no obvious signs of bacterial growth (no color change of phenol red indicator or no increase in turbidity above that contributed by the inoculum) were recorded as negative. In many experiments, all tubes which showed no macroscopic evidence of growth were subcultured 11 or 30 days after challenge. The objectives of this investigation were, however, concerned mainly with inhibition of growth, regardless of whether inhibition was due to bacteriostasis or to death of the challenge cultures.

Most results were obvious after 11 days of incubation, but occasionally some tubes which appeared negative at 11 days contained visible growth after 30 days at room temperature.

[1] Difco Laboratories, Detroit, Mich.

TABLE V.—ADSORPTION OF BEC BY PERTUSSIS VACCINE

Vaccine	BEC, p.p.m. Added	BEC, p.p.m. Found
Tetanus toxoid	25	18
Tetanus toxoid with polio vaccine	25	19
Diphtheria toxoid with polio vaccine	25	19.5
Tetanus toxoid, diphtheria toxoid, and pertussis vaccine with polio vaccine (DPT Polio)	25	7
Pertussis vaccine	50	15

RESULTS

Benzethonium Chloride Content of Vaccines.—Table II shows the BEC content found in several commercially produced vaccines.

Table III shows that if BEC is added to poliomyelitis vaccine only about 80% can be found upon analysis when the vaccine is dispensed in 10-ml. amounts in multiple-dose vials and even less is found in 1-ml. ampuls. The reason for less than complete recovery of the BEC is probably adsorption on the glass as found by Fogh, *et al.*, with cetylpyridinium chloride (14). The smaller containers would have a larger *surface of glass/volume of liquid* ratio than the 10-ml. vials.

Table IV shows that BEC also disappears when poliomyelitis vaccine is converted to a multivalent vaccine (DPT Polio) by the addition of four volumes of combined diphtheria toxoid, tetanus toxoid, and pertussis vaccine to 100 volumes of poliomyelitis vaccine. The three additives had been preserved by the addition of at least 25 p.p.m. of BEC, so dilution of the BEC in the poliomyelitis vaccine was not a factor in producing the low levels found.

Apparently either the toxoids or pertussis cells adsorb or otherwise change benzethonium chloride. Table V shows that the presence of pertussis cells is a cause of failure to recover all of the added

TABLE VI.—PRESERVATIVE ACTIVITY OF BEC IN HB597 MEDIUM

BEC, p.p.m.	Challenge Culture, Dilution	No. tubes with growth / No. tubes challenged: Bacteria	Yeasts and Molds
0	None	13/13	7/7
	10^{-3}	13/13	6/7
	10^{-6}	13/13	2/7
24	None	11/13	6/7
	10^{-3}	11/13	0/7
	10^{-6}	11/13	0/7

TABLE VII.—ANTIBIOTIC CONCENTRATION IN POLIOMYELITIS FLUIDS DURING VARIOUS STAGES OF PRODUCTION

Tissue Culture Fluid	Antibiotic Concn. (mcg. per ml.)		
	Polymyxin	Neomycin	Streptomycin
Live virus fluid	<2	10	180
Inactivated 7 days	<2	14	170
Inactivated 12 days	0	10	135

benzethonium chloride. The toxoids do not remove appreciable amounts of BEC.

Preservative Activity of BEC.—To test the antimicrobial activity of BEC in systems uncomplicated by the presence of antibiotics normally found in poliomyelitis vaccine, BEC was added to antibiotic-free HB597 medium and the 4 ml. of medium was challenged with 0.1 ml. of the challenge cultures previously described. Table VI shows that 24 p.p.m. of BEC has little antibacterial activity but has some antifungal activity against the lower concentrations of fungal cells. The two bacterial cultures inhibited by the BEC were *Staphylococcus aureus*.

As stated previously, BEC is not the only preservative in poliomyelitis vaccine. In production of vaccine the HB597 medium used in the tissue culture had added to it 200 units of streptomycin, 20 units of polymyxin, and 10 units of neomycin per ml. Table VII shows that the polymyxin is completely destroyed during the production of vaccine but that the neomycin and streptomycin are stable.

As a check on the preservative power of the residual antibiotics in poliomyelitis vaccine, 50 strains of *Staphylococcus aureus* were obtained from a clinical laboratory. It was assumed (but not checked) that from such a source many of the organisms would be resistant to antibiotics. Fifty test tubes were charged with poliomyelitis vaccine in which the formaldehyde had been neutralized and to which no preservative had been added. Each tube was inoculated with about 10^8 cells of a different strain of *S. aureus*. No growth was observed in any of the tubes. The residual antibiotics were evidently effective preservatives against most strains of *S. aureus*.

Table VIII shows that the antibiotics remaining in finished poliomyelitis vaccine possess some activity against the test organisms and that this activity is not increased appreciably by BEC.

Experiments similar to those depicted in Table VIII but with tubes incubated at 4° showed that many of the G-negative bacteria were able to grow at refrigerator temperature if the incubation period was extended to 2 months.

To test the effect of the addition of polymyxin to finished poliomyelitis vaccine which contained BEC, a vaccine which contained only streptomycin (200 mcg. per ml.) was used. Three separate experiments were run. The aggregated results (Table IX) show that polymyxin and neomycin do not increase the preservative activity obtained by streptomycin alone.

Table IX, like Table VIII, shows that the addition of BEC to vaccine which contains only streptomycin gives a slight increase in preservative activity. However, when polymyxin and neomycin as well as BEC were added to the vaccine containing streptomycin there was a definite increase in preservative activity. Apparently there is some synergistic or additive effect between polymyxin and BEC. (Compare with Table VIII.)

TABLE VIII.—EFFECT OF BEC ON THE PRESERVATIVE ACTIVITY OF POLIOMYELITIS VACCINE[a] CONTAINING RESIDUAL STREPTOMYCIN AND NEOMYCIN

Challenge Culture (bacteria), Dilution	No. tubes with growth / No. tubes challenged, BEC concn., p.p.m. 0	8	22
None	13/13	13/13	9/13
10^{-3}	7/13	7/13	5/13
10^{-6}	3/13	2/13	4/13

[a] For maldehyde neutralized with sodium bisulfite.

TABLE X.—DISAPPEARANCE OF POLYMYXIN B FROM POLIOMYELITIS VACCINE DURING STORAGE

Time of Storage, wks.	Concn. Polymyxin, p.p.m. 4°C.	25°C.
0	20	20
2		11.2
5	21	
6		4
9	12.5	

TABLE IX.—ANTIBIOTICS AND BEC AS ANTIBACTERIAL AGENTS IN POLIOMYELITIS VACCINE[a]

Antibiotic Mixture, mcg. per ml.		BEC, p.p.m.	Challenge Cultures (bacteria), Dilution	Growth[b] Challenged	Positive, %
Streptomycin	200		None	36/37[c]	97
Polymyxin	0	0	10^{-3}	26/38	68
Neomycin	0		10^{-6}	16/38	42
Streptomycin	200		None	36/39	92
Polymyxin	0	26	10^{-3}	18/39	46
Neomycin	0	(range 24–29)	10^{-6}	12/39	31
Streptomycin	200		None	37/39	95
Polymyxin	20	0	10^{-3}	26/39	70
Neomycin	8		10^{-6}	15/39	39
Streptomycin	200		None	30/36	83
Polymyxin	20	26	10^{-3}	11/36	31
Neomycin	8	(range 24–29)	10^{-6}	4/36	11

[a] Formaldehyde neutralized with sodium bisulfite. [b] Number of tubes with growth/number of tubes challenged. [c] Aggregate of three separate experiments using cultures C-1 to C-16.

TABLE XI.—LOSS OF PRESERVATIVE EFFECT RESULTING FROM THE NEUTRALIZATION OF FORMALDEHYDE IN SALK VACCINE[a] WITH SODIUM BISULFITE

Medium	Free Formaldehyde, p.p.m. (modified Nash test)	Challenge Culture, Dilution	No. tubes with growth / No. tubes challenged: Bacteria	Yeasts and Molds
HB597	4.0	None	13/13	7/7
		10^{-3}	13/13	6/7
		10^{-6}	13/13	5/7
Lot 155, polio vaccine	72.8	None	2/13	6/7
		10^{-3}	0/13	2/7
		10^{-6}	0/13	1/7
Lot 155, polio vaccine with bisulfite	7.5	None	11/13	7/7
		10^{-3}	3/13	5/7
		10^{-6}	0/13	6/7

[a] BEC not added.

TABLE XII.—ANTIFUNGAL ACTIVITY OF BEC IN POLIOMYELITIS VACCINE CONTAINING ANTIBIOTICS AND NON-NEUTRALIZED FORMALDEHYDE

Medium	Challenge Culture, Dilution	No. tubes with growth / No. tubes challenged: Bacteria	Yeasts and Molds
HB597	None	13/13	7/7
	10^{-3}	13/13	6/7
	10^{-6}	13/13	2/7
Polio vaccine	None	1/13	6/7
	10^{-3}	0/13	2/7
	10^{-6}	0/13	1/7
Polio vaccine with 20 p.p.m. benzethonium chloride	None	1/13	3/7
	10^{-3}	0/13	0/7
	10^{-6}	0/13	0/7

It appeared desirable to increase the preservative activity in poliomyelitis vaccine, either by increasing the concentration of BEC or by adding polymyxin to the inactivated vaccine just before packaging. Unfortunately, increasing the concentration of BEC to 33 p.p.m. caused a precipitate in the vaccine and as shown in Table X, polymyxin is unstable even at 4° and would be valueless in vaccines stored for several months.

The above results indicated that BEC added little antibacterial activity to that obtained from the residual antibiotics in Salk vaccine. It did, however, confer some antimycotic activity (Table VI).

In contrast to the inadequate antibacterial activity of BEC it was found that formaldehyde was an excellent preservative in poliomyelitis vaccine. Table XI shows that the neutralization of formaldehyde by bisulfite decreases markedly both the antibacterial and antimycotic activity in poliomyelitis vaccine.

The addition of BEC to poliomyelitis vaccine containing residual formaldehyde does not materially increase the antibacterial effect on the test organisms used but, as Table XII shows, the BEC increases the antifungal activity of the preservatives in the poliomyelitis vaccine.

DISCUSSION

The objective of this research was to determine the reason for growth of bacterial and fungal contaminants in poliomyelitis vaccine preserved by the addition of 25 p.p.m. of BEC. McLean (1), Schuchardt, *et al.* (15), and Schuchardt (16) have shown that this compound, added as a preservative to poliomyelitis vaccine, did not significantly damage the antigenicity of the vaccine. They stated that it should be used preferably in vaccines to which bisulfite had not been added, but they failed to present data concerning its efficacy as a preservative with or without neutralization of formaldehyde.

The work described in this report furnishes two reasons for the failure of BEC to give adequate preservation to poliomyelitis vaccine and DPT Polio vaccine. The most important reason is that BEC at 25 p.p.m. or less is not a very effective inhibitor of G-negative bacteria. Other workers have reported this deficiency (2, 3). The other apparent reason is the loss of BEC, probably because of adsorption on glass or bacterial cells. The phenomenon of adsorption on glass has been reported for other quaternary ammonium compounds by Fogh, *et al.* (14).

The challenge dose used in these experiments was, in our opinion, severe. Not only were large numbers of organisms used (up to 10^8 viable cells), but also many of the cultures were isolated from contaminated vaccines. The necessity of using a severe challenge is evident; the desirability of using contaminants from biologicals has been emphasized by Pittman and Feeley (17). The use of less severe challenge doses (Table VI) shows clearly the lack of ability of BEC to inhibit even small numbers (10^2 to 10^5 cells) of G-negative bacteria.

There is little or no need for BEC as an inhibitor of G-positive bacteria in poliomyelitis vaccine and (as stated above) its ability to inhibit many G-negative bacteria is questionable. As an antifungal agent it does serve some purpose. Therefore, a preservative which has the antifungal activity of BEC and the antibacterial activity of formaldehyde is still needed in poliomyelitis vaccine, especially if the formaldehyde has been inactivated by bisulfite. Such a compound has been found and its effectiveness will be the subject of a later report.

SUMMARY

Benzethonium chloride (BEC) is adsorbed on glass and on bacterial cells.

BEC alone does not inhibit the growth of sev-

eral G-negative bacteria but does inhibit the growth of G-positive bacteria, yeasts, and molds.

BEC adds little antibacterial effect to the residual antibiotics present in poliomyelitis vaccine but does contribute antimycotic activity.

Formaldehyde, if not neutralized by bisulfite, is highly antibacterial and exhibits some antimycotic activity.

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Oxidation of Sulfurous Acid Salts in Pharmaceutical Systems

By LOUIS C. SCHROETER

Many aqueous, oxygen-labile pharmaceutical systems may be effectively protected by the antioxidant activity of sulfurous acid salts. The sulfite system is inordinately sensitive to trace amounts of heavy metal catalysts and a wide variety of organic compounds which act as oxidation inhibitors. Mechanism of sulfite oxidation in the presence of known inhibitors at levels normally encountered in pharmaceutical formulations appears to involve heavy metal catalysts. At the concentration (p.p.m.) of heavy metals normally present in formulations, the rate of oxidation and antioxidant activity is directly dependent upon the heavy metal concentration. However, a slow but measurable rate of sulfite oxidation occurs in systems especially purified to remove metal ions. The copper-catalyzed oxidation of sulfurous acid salts in buffered systems containing ethanol as an inhibitor may be described by first-order kinetics. The pH profile of the absolute initial rate of the copper-catalyzed oxidation has been experimentally determined and found to be in reasonable agreement with a theoretically derived curve.

ORGANIC COMPOUNDS in very low concentrations (10^{-6} M) appear to inhibit the rate of oxidation of aqueous solutions of sulfurous acid salts (1). This inhibitory action has been demonstrated by many compounds representing diverse structural features and functional groupings—typical drug molecules may be expected to retard the rate of sulfite oxidation (2). This fact may at first seem paradoxical since it is well known that sulfurous acid salts are employed as pharmaceutical antioxidants (3). Pharmaceutical systems generally contain much more drug than antioxidant: the molar concentration of drug may be as much as two orders of magnitude greater than that of the antioxidant.

Effectiveness of sulfurous acid salts as pharmaceutical antioxidants in most aqueous systems appears to depend on their avidity for free radicals such as OH or simply on the ease with which they are oxidized in comparison with most autoxidizable drugs (3). However, this reveals little about the mechanism of sulfite oxidation in the presence of known inhibitors.

Inhibition of the rate of sulfite oxidation may be described by an equation (1–3)

$$-\frac{d(S_t)}{dt} = \frac{k_1(S_t)A}{B + M} \qquad \text{(Eq. 1)}$$

where S_t is the concentration of total sulfurous acid species, k_1 is the specific rate constant for the uninhibited reaction, M is the molar concentration of additive, and A and B are constants generally of the magnitude 10^{-5}. The specific rate constant, k_1, for the air oxidation (atmospheric pressure, 25°) of pure aqueous sulfite is 2.9×10^{-3} seconds^{-1} under conditions such that the diffusion or dissolution rate of oxygen is not rate limiting; the copper-catalyzed (10^{-6} M Cu^{2+}) reaction yields an apparent rate constant of 5.1×10^{-3} seconds^{-1}. Inhibition of sulfite oxidation by a variety of compounds over

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a wide range (10^{-3} to 10^{-7} M) of inhibitor concentration has been satisfactorily described by Eq. 1 or others of similar form (1–4). Activity of inhibitors in breaking sulfite radical chains appears to be proportional to the inhibitor concentration in dilute ($\leq 10^{-3}$ M) solutions of inhibitor and independent of the primary chain activation. The thermal (dark) and photo-induced reactions of sulfite in the presence of inhibitors both proceed by a radical process and may be described by an equation in which the rate is inversely proportional to the sum of a constant and the molar concentration of inhibitor, *i.e.*, Eq. 1, holds for dilute (10^{-3} to 10^{-7} M) solutions of the inhibitor (5). Chain termination of the sulfite radical in the presence of an inhibitor has been shown (6) to yield traces of oxidized inhibitor. For example, oxidation of sulfite in the presence of benzyl alcohol or isopropyl alcohol yields small quantities of benzaldehyde and acetone, respectively. The inhibitor apparently provides an alternate reaction pathway to the chain propagation process (7) and is itself oxidized to a noncarrier product. This would correspond to a bimolecular reaction with a first-order dependence on inhibitor concentration and first-order dependence on the radical chain carrier. The oxidation of inhibitors through their interaction with radicals generated in systems such as sulfite corresponds to Jorissen's "Induced Oxidation" (8).

Reliable data on the rate of oxidation of sulfite in the presence of high concentrations ($>10^{-2}$ M) of inhibitors are not available. Jorissen (8) reports values for sodium sulfite oxidation in the presence of relatively high concentrations (0.25 M) of inhibitors in pure oxygen. The times required for essentially complete oxidation of sulfite in the presence of 0.25 M mannitol and 0.25 M *n*-propanol were 13 days and 18 days, respectively. The

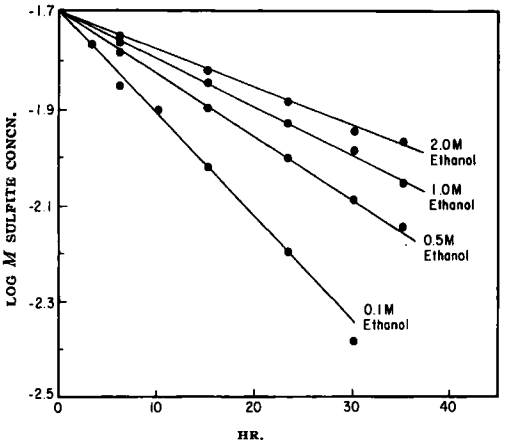

Fig. 1.—Air oxidation of 0.02 M sodium sulfite in the presence of Cu (II) (1×10^{-6} M) and ethanol at 25°C.

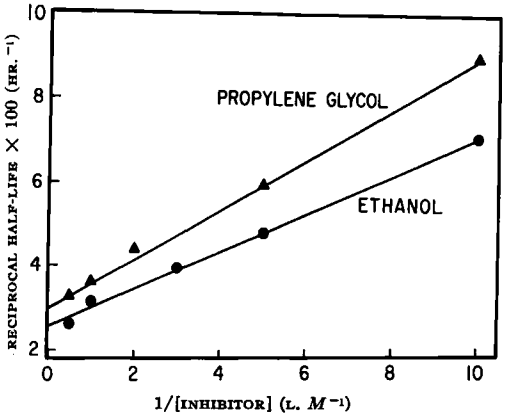

Fig. 2.—Reciprocal half-life for the cupric ion (1×10^{-6} M) catalyzed air oxidation of sodium sulfite (0.02 M) as a function of reciprocal inhibitor concentration at 25°C.

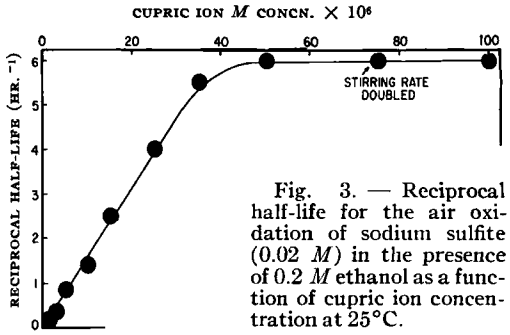

Fig. 3. — Reciprocal half-life for the air oxidation of sodium sulfite (0.02 M) in the presence of 0.2 M ethanol as a function of cupric ion concentration at 25°C.

theoretical value for the time required for 95% ($t_{95\%}$) oxidation of sulfite in the presence of 0.25 M inhibitor may be approximated with

$$t_{95\%} = \frac{2.303}{k_1} \log \frac{100}{5} = \frac{2.99}{k_1} \quad \text{(Eq. 2)}$$

where k_1 is the specific rate constant for the oxidation in pure oxygen in the presence of 0.25 M inhibitor calculated with Eq. 1 using Fuller and Crist's (9) value (1.3×10^{-2} seconds^{-1}) for the noninhibited rate.

$$k_1 = \left(\frac{10^{-5}}{10^{-5} + 0.25}\right) 1.3 \times 10^{-2} \text{ sec.}^{-1} = 5.2 \times 10^{-7} \text{ sec.}^{-1}$$

These approximate calculations yield a value of about 67 days or almost 2 months for the time required for 95% of sulfite to oxidize ($t_{95\%}$) in the presence of 0.25 M inhibitor and pure oxygen. The predicted value, $t_{95\%}$, obtained with Eq. 1 is about five times greater than that found experimentally (13–18 days).

Failure of Eq. 1 to describe the effect of inhibitors

TABLE I.—HALF-LIVES OF THE AIR OXIDATION OF 0.02 M SODIUM SULFITE IN THE PRESENCE OF 0.2 M ETHANOL AT 25°

Additive	Solution pH—Initial	Solution pH—Final[a]	Half-Life
5×10^{-5} M Cu(II)	9.2	8.0	0.167 hr.
1×10^{-3} M NaCN	9.3	8.3	1525 hr.

[a] pH measured after two half-life periods.

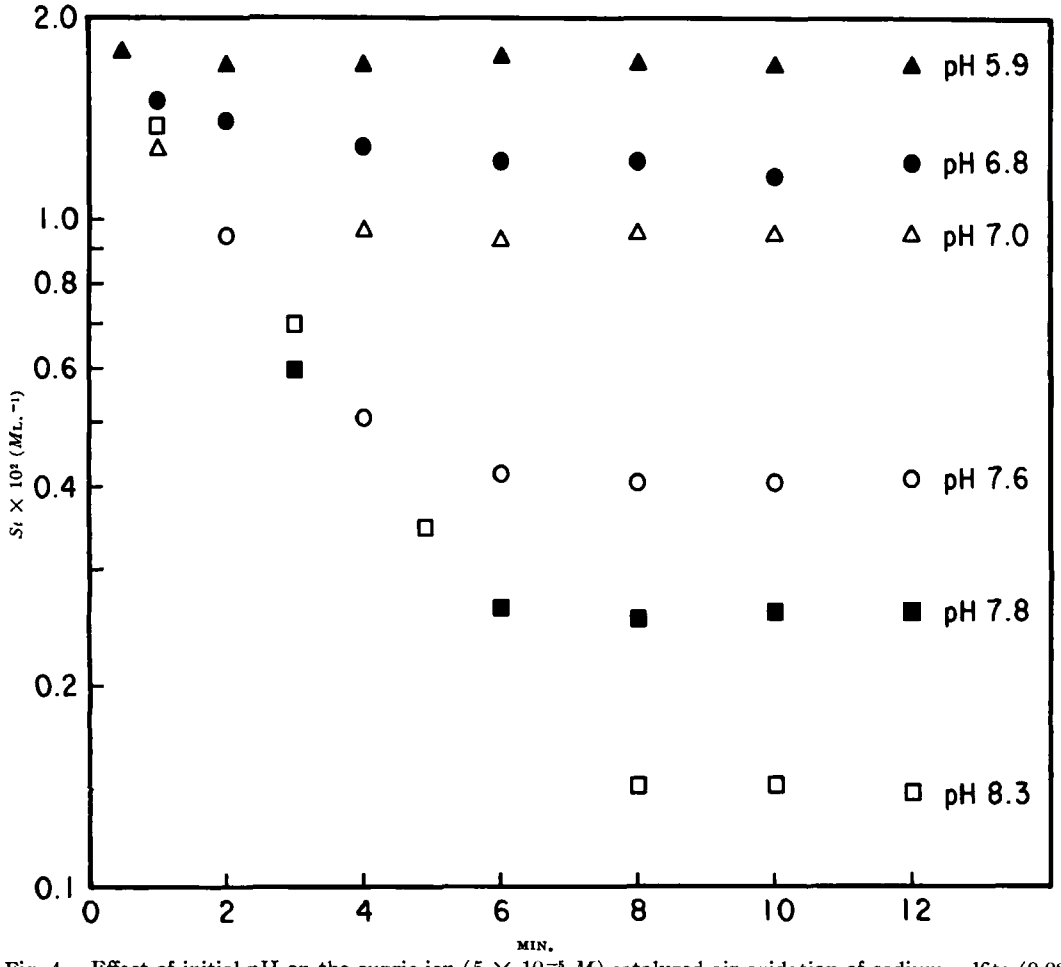

Fig. 4.—Effect of initial pH on the cupric ion (5×10^{-5} M) catalyzed air oxidation of sodium sulfite (0.02 M) in the presence of 0.1 M ethanol at 25°C.

under conditions described above is not wholly unexpected nor without precedent in radical reactions. The form of Eq. 1 is determined by the nature of the chain-breaking process and not by the initiation process (10). The presence of inhibitors generally alters only the rate of chain propagation and not the rate of radical initiation. Thus, the observed decrease in total sulfite which occurs in oxidizing systems containing inhibitors may be accounted for if radial initiation or some fraction thereof leads to oxidized product (sulfate); the radical initiating process becomes the rate-determining step in the overall oxidation scheme when the rate of chain propagation is decreased or, in equivalent terms, when the chain length is reduced. Existence of a measurable reaction rate in radical mediated processes in the presence of overwhelming amounts of inhibitor has been described as the "completely inhibited" rate (10). In the case of nitric oxide inhibition of alkyl free radical gas reactions, the completely inhibited or residual reaction rate has been interpreted in terms of an alternate nonradical mechanism (11); however, there is some evidence of free radicals in these systems (12). Heavy metal catalysis of sulfite oxidation allows another possible mechanism for radical initiation; this pathway for radical initiation may be important in systems containing inhibitors. The catalytic effect of copper ions on sulfite oxidation may be detected in concentrations as low as 10^{-6} M. This is within the permissible range of parts per million of heavy metal contaminants for many compounds of pharmaceutical interest (13); it is a reasonable assumption to consider that most complex pharmaceutical formulations contain catalytic (1 p.p.m.) amounts of heavy metals. It is the

Table II.—Effect of Initial pH on the Cupric Ion (5×10^{-5} M) Catalyzed Air Oxidation of Sodium Sulfite (0.02 M) in the Presence of 0.1 M Ethanol at 25°

pH		Initial[b] $[HSO_3^-] \times 10^2$	Final[c] $S_t \times 10^2$
Initial	Final[a]		
5.9	4.5	1.78	1.70
6.8	4.5	1.32	1.20
7.0	4.7	1.10	0.95
7.6	4.9	0.47	0.40
7.8	5.6	0.32	0.25
8.3	6.0	0.12	0.14

[a] Observation recorded after 10–12 minutes—the plateau region shown in Fig. 4. [b] Calcd: $[HSO_3^-] = S_t - SO_3^{2-} = S_t - S_t/[H^+]/(K_{a2}') + 1$; apparent second ionization constant of sulfurous acid: 8.2×10^{-8}. [c] Total sulfurous acid species, $S_t = [HSO_3^-] + [SO_3^{2-}]$, determined iodometrically.

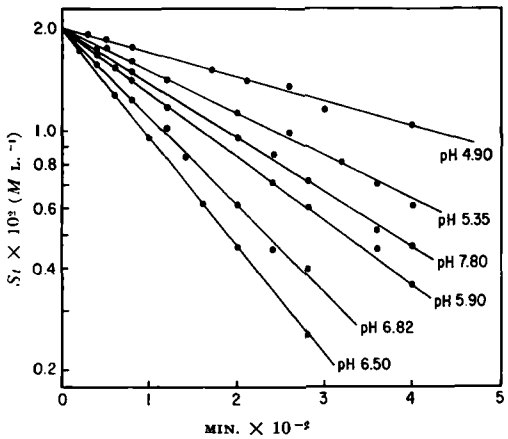

Fig. 5.—Cupric ion (5×10^{-5} M) catalyzed air oxidation of sulfurous acid specie ($S_t = 0.02$ M) in the presence of 0.2 M ethanol at constant pH (McIlvaine buffers 0.3 M; total ionic strength adjusted to 1.0 with KCl).

purpose of this study to describe some of the kinetic and mechanistic aspects of sulfite oxidation in the presence of a known inhibitor (ethanol) and in systems containing both inhibitor and catalyst.

EXPERIMENTAL

Water used in these studies was distilled twice from a Pyrex apparatus and stored not longer than 6 hours under nitrogen atmosphere prior to use. Reagent grade sodium sulfite was recrystallized twice from water. Ethanol was distilled twice from a Pyrex apparatus. All other materials were of reagent grade and used without further purification unless otherwise specified. Rapid ($t^1/_2 \leq 5$ minutes) oxidations were carried out in a vessel described in a prior communication (1). Slower reactions were studied by vigorously agitating the solution in a flask: 25 ml. of solution contained in a 500-ml. Erlenmeyer flask was shaken at a constant rate with a Burrell wrist action shaker[1] in a thermostatted room in which temperature was controlled at 25 ± 1°. Total sulfurous acid content of solutions was determined iodometrically. The pH of each solution was measured at 25 ± 0.05° with a Beckman GS pH meter.

RESULTS AND DISCUSSION

The copper-catalyzed air oxidation of aqueous-ethanol solutions of sodium sulfite exhibits an apparent first-order dependency on total sulfurous acid species as shown in Fig. 1. The apparent first-order rate constant decreases with increasing concentrations of ethanol: 0.1 M ethanol, k = 0.050 hours^{-1}; 0.5 M ethanol, k = 0.030 hours^{-1}; 1.0 M ethanol, k = 0.023 hours^{-1}; 2.0 M ethanol, k = 0.018 hours^{-1}.

Selection of ethanol as the chain propagation inhibitor was made on the basis of its importance in pharmaceutical formulations and the ease with which it can be distilled free from heavy metal contaminants. Cupric ion ($1 \times 10^{-6} M$) catalyst, which was added to each system immediately prior to oxidation, corresponded to less than 0.1 p.p.m. as Cu(II). Total heavy metal content of the oxidation system was not directly determined; however, kinetic oxidative studies with varying concentrations of sodium sulfite in water-ethanol indicated the presence of less than 10 p.p.b. copper ion.

[1] Burrell Corp., Pittsburgh, Pa.

TABLE III.—APPARENT FIRST-ORDER RATE CONSTANTS FOR THE COPPER CATALYZED OXIDATION OF SULFUROUS ACID SALTS AT CONSTANT pH IN THE PRESENCE OF 0.2 M ETHANOL[a]

pH[b]	$\left(\frac{\sqrt{(H^+)}}{K_s' + (H^+)}\right)^c$	$k \times 10^2$ (min.$^{-1}$)
4.90	276	1.65
5.35	465	2.88
5.90	738	4.32
6.50	984	7.08
6.82	962	5.78
7.80	465	3.84

[a] Solutions buffered with 0.3 M McIlvaine buffer; total ionic strength of the system adjusted to 1.0. [b] pH of solutions determined at 25° prior, during, and after oxidation (three half-lives) with Beckman GS pH meter did not vary more than 0.05 pH unit from value shown. [c] Experimentally determined apparent second ionization constant for sulfurous acid, $K_s' = 2.55 \times 10^{-7}$ (25°, $\mu = 1.0$).

Dependence of the copper-catalyzed (1×10^{-6} M) oxidation rate of sodium sulfite on inhibitor concentration is shown in Fig. 2. The linear relationship between the reciprocal half-life for the copper-catalyzed reaction and the reciprocal of the concentration of added inhibitor clearly demonstrates that significant oxidation of the thio compound takes place in the presence of overwhelming amounts of inhibitor when copper is present. The inhibitor appears to have little or no effect on radical initiation; it appears to function primarily by decreasing chain length. The heavy metal catalyzed oxidation of sulfurous acid salts in the presence of high concentrations of inhibitor is especially significant since this simulates pharmaceutical systems in which the compounds are utilized as antioxidants.

The rate of oxidation of sodium sulfite in the presence of 0.2 M ethanol appears to be directly proportional to the concentration of added cupric ion over the concentration range 0 to 30×10^{-6} M Cu(II) as shown in Fig. 3. However, a slow but measurable rate of oxidation of sodium sulfite takes place in the presence of an inhibitor even in the absence of added copper ion. This uncatalyzed rate of sulfite oxidation in the presence of an inhibitor may be approximated with Eq. 1 assuming $A = B = 10^{-5}$ and using the specific rate constant for the oxidation of sodium sulfite in air, $k = 2.8 \times 10^{-3}$ seconds^{-1}. For a sodium sulfite solution containing 0.2 M ethanol as an inhibitor, Eq. 1 predicts an approximate first-order rate constant for the inhibited oxidation: $k_1 = 5 \times 10^{-4}$ hours^{-1}; in terms of half-life, $t^1/_2 \cong 1400$ hours. This theoretical value agrees reasonably well with the experimentally determined half-life (1525 hours) for sulfite oxidation in the presence of 0.2 M ethanol and 1×10^{-3} M sodium cyanide as shown in Table I. Since the uncatalyzed reaction rate is four orders of magnitude less than the maximum copper-catalyzed rate, it is not detected as an intercept value on a rate plot such as Fig. 3. At higher concentrations ($> 40 \times 10^{-5}$ M) of the metal

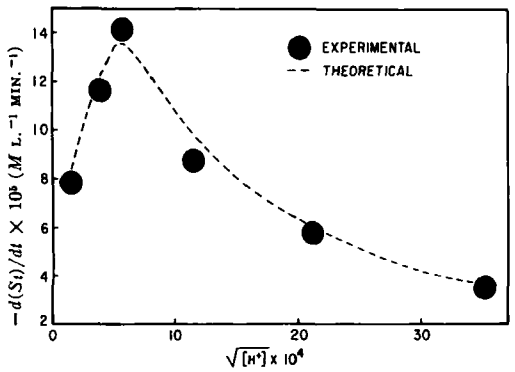

Fig. 6.—Initial rate of loss of total sulfurous acid species ($-d(S_t)/dt$) as a function of square root of the hydrogen ion concentration at 25°C. Initial solution composition: 0.02 M total sulfurous acid species, 0.2 M ethanol, 5×10^{-5} M Cu (II) in 0.3 M buffers. Ionic strength unity ($\mu = 1.0$). Theoretical line calculated with Eq. 4.

ion, the reaction appears to reach a limiting rate corresponding to a half-life for the oxidation of about 10 minutes. Doubling the stirring rate to increase the dissolution of oxygen does not apparently affect the limiting rate under these experimental conditions. This rate-limiting step may be related to the rate of reduction of Cu(II) or its regeneration.

Effect of initial solution pH on the copper-catalyzed oxidation of sulfurous acid salts in aqueous-ethanol solution is shown in Fig. 4. The initial pH of each of these solutions was adjusted to the indicated values with appropriate amounts of standard sodium hydroxide solution; the test solutions contained no other buffer species. Rapid initial oxidation occurred in all solutions at initial pH values from 5.9 to 8.3. The oxidation rates appeared to reach a plateau region in which little or no change occurred during the time of observation. The oxidation of bisulfite proceeds with the production of the more acidic sulfate species and an increase in the hydrogen ion concentration. The apparent effect is to decrease the total sulfurous acid species (HSO_3^- and SO_3^{2-}) wholly at the expense of sulfite as shown in Table II. This phenomenon has been discussed in a previous publication (1). The increase in hydrogen ion concentration in unbuffered oxidizing systems decreases the rate of oxidation in accordance with

$$-\frac{d(S_t)}{dt} = g\frac{(HSO_3^-)}{\sqrt{(H^+)}} \qquad \text{(Eq. 3)}$$

Oxidation of sulfurous acid salts in pharmaceutical systems usually takes place under conditions of constant pH since these compounds are added in small amounts and rarely represent the major buffer species. Oxidation of sulfurous acid salts in strongly buffered systems follows apparent first-order kinetics as shown in Fig. 5. The oxidation was studied in duplicate over about 90% of the reaction course (three half-lives). The relationship between the pH and the slopes of the lines representing the specific first-order rate constants suggests the presence of a maximum in the pH-rate constant profile. Table III indicates that the rate constants increase up to pH 6.5 and decrease at higher pH values. This unique dependency of the oxidation rate on hydrogen ion concentration may be satisfactorily explained by a modified form of Eq. 3. The concentration of hydrogen sulfite may be described in terms of total sulfurous acid specie added to the system, S_t

$$[HSO_3^-] = \frac{S_t(H^+)}{K_s' + (H^+)} \qquad \text{(Eq. 4)}$$

Substituting Eq. 4 into Eq. 3 gives

$$-\frac{d(S_t)}{dt} = g\frac{\frac{S_t(H^+)}{K_s' + (H^+)}}{\sqrt{(H^+)}} = g \cdot S_t \frac{\sqrt{(H^+)}}{K_s' + (H^+)} \qquad \text{(Eq. 5)}$$

The relationship shown above predicts a maximum oxidation rate when $[H^+] = K_s'$ since the terms $(\sqrt{(H^+)}/K_s' + (H^+))$ is maximal at this hydrogen ion concentration.

This further supports Abel's theoretical predictions (14) which were found to describe limited experimental data derived from unbuffered oxidizing sulfite systems. The rather good agreement between experimentally determined absolute initial rate of oxidation in buffered sulfite systems and the theoretical curve from Eq. 5 is shown in Fig. 6. The experimental maximum rate occurs at a hydrogen ion concentration of 3.16×10^{-7} which shows reasonable agreement with the theoretical value 2.55×10^{-7}. The latter value represents the experimentally determined apparent second ionization constant of sulfurous acid at 25° in buffer of total ionic strength equal to unity.

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Binding of Organic Electrolytes by a Nonionic Surface-Active Agent

By ARTHUR R. HURWITZ†, PATRICK P. DELUCA‡, and H. B. KOSTENBAUDER

Although possible consequences of combining ionic pharmaceuticals with surface-active agents of opposite charge are generally recognized, there has been little or no consideration of possible interactions between drug ions and nonionic surface-active agents. In the present study, cations such as chlorpromazine, promethazine, tetracaine, methylrosaniline, and dodecylpyridinium, and anions such as naphthalenesulfonate and methyl orange were bound to the nonionic surface-active agent polysorbate 80. The degree of interaction in some cases is sufficient to suggest that polysorbate 80 might have considerable influence on the stability and availability of ionic drugs in pharmaceutical formulations.

THE USE OF nonionic surface-active agents in the formulation of various pharmaceutical dosage forms is often a routine procedure. Their presence in pharmaceuticals sometimes results in incompatibilities—notably, the inactivation of some antimicrobial agents which are commonly used in pharmaceuticals for their preservative activity (1–16). In the majority of examples of incompatibility which have previously been described, the agent which is bound to the surfactant is primarily in molecular or nonionized form; interaction with the surfactant has generally been explained on the basis of either the formation of specific molecular complexes or the preferential solubility in the surfactant micelle. Persons engaged in pharmaceutical formulation are generally cognizant of possible incompatibilities of this nature.

Although textbooks devote considerable discussion to incompatibilities which might arise from the combination of an ionic surfactant with a drug or germicide of opposite charge, there is usually no suggestion of possible incompatibility or inactivation which might arise from the combination of an ionic drug or germicide with a nonionic surfactant. Recent observations in these laboratories indicated that organic ions, such as quaternary ammonium germicides, can be strongly bound by nonionic surfactants (16). These observations suggest that such generally unrecognized incompatibilities might exist in many pharmaceutical systems and might markedly influence the release of a drug from a dosage form or might alter the stability of a drug. The present study was designed to investigate the possible association of various cations and anions with a typical nonionic surface-active agent, polysorbate 80.[1] The existence of a significant degree of interaction between ionic drugs and nonionic surfactants might require a re-evaluation of the routine use of nonionic surface-active agents in pharmaceutical formulation.

EXPERIMENTAL

Reagents.—Ephedrine hydrochloride N.F.; sulfathiazole sodium N.F.; methylrosaniline chloride U.S.P.; promethazine hydrochloride U.S.P.[2]; methapyriline hydrochloride N.F.[3]; diphenhydramine hydrochloride U.S.P.[4]; chlorpromazine hydrochloride U.S.P.[5]; tetracaine hydrochloride U.S.P.[6]; cetylpyridinium chloride U.S.P.[7]; polysorbate 80, a commercial sample; sodium salt of 2-naphthalene sulfonic acid, recrystallized from water; dodecylpyridinium bromide, laboratory prepared; were used. All other chemicals were reagent grade.

Method for Detecting Interaction.—Equilibrium dialysis was employed to detect possible drug–nonionic surface-active agent interaction. The technique was similar to that described previously by Patel and Kostenbauder (17) in their study of the interaction of methyl and propyl *p*-hydroxybenzoate with polysorbate 80. This method involves equilibration of two solutions across a semipermeable membrane, one solution containing the surface-active agent and the other solution containing only the drug. Nylon membranes were used in this study, these membranes being selected to permit the drug to come to equilibrium in both solutions but to prevent passage of the nonionic surface-active agent.

The nylon membranes were cut to give bags which held 20 ml. of solution. The bags, when filled with the solution of the ionic agent being studied, were tied and placed in wide mouth,

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[1] Polyoxyethylene 20 sorbitan monooleate is marketed as Tween 80 by Atlas Powder Co., Wilmington, Del.
[2] Marketed as Phenergan Hydrochloride by Wyeth Laboratories, Philadelphia, Pa.
[3] Marketed as Histadyl Hydrochloride by Eli Lilly and Co., Indianapolis, Ind.
[4] Marketed as Benadryl Hydrochloride by Parke, Davis and Co., Detroit, Mich.
[5] Marketed as Thorazine Hydrochloride by Smith Kline and French Laboratories, Philadelphia, Pa.
[6] Marketed as Pontocaine Hydrochloride by Winthrop Laboratories, New York, N. Y.
[7] Marketed as Ceepryn by The William S. Merrell Co., Cincinnati, Ohio.

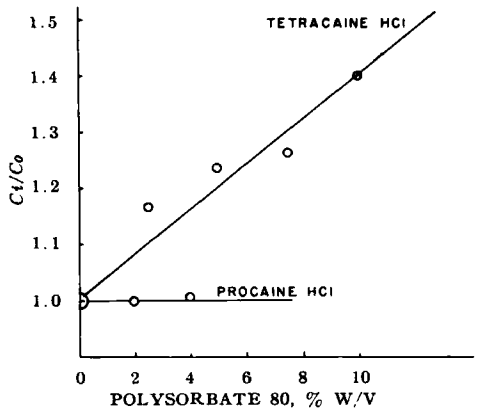

Fig. 1.—Binding of tetracaine hydrochloride by polysorbate 80 at 30°.

screw-cap, glass bottles containing 60 ml. of a polysorbate 80 solution with a concentration of ionic agent equal to that in the internal phase. By starting with equal concentrations of drug in both phases, the equilibration or agitation time could be greatly reduced. Also, shorter agitation time decreased the possibility of membrane breakage. The bottles were then tightly closed, using a piece of polyethylene film between the cap and the bottle top to prevent leakage. The bottles were agitated in a constant temperature water bath at 30° for 2 to 6 days. Aliquot portions were then taken from both the internal and external phases and assayed spectrophotometrically for the ionic agent, using a Beckman DU spectrophotometer and an appropriate blank solution in each case. A few agents were studied in the presence of potassium chloride to determine the effect of salt on the interaction, and sodium bisulfite was added to solutions of promethazine and chlorpromazine to retard oxidation. The spectrophotometric assays of methyl orange and methylrosaniline chloride were performed on the Beckman model B spectrophotometer. The addition of polysorbate 80 (2% w/v) caused a shift in the absorption maximum of methyl orange from 465 mμ to 425 mμ, and the maximum for methylrosaniline chloride was shifted from 595 mμ to 600 mμ. The polysorbate-containing solutions of these agents were always assayed by adjusting the final polysorbate concentration to 2%, then determining the absorbance at the shifted maximum.

The extent of binding is represented as C_i/C_o, where C_i represents the total concentration of organic electrolyte on the polymer-containing side of the membrane, and C_o is the concentration on the nonpolymer-containing side. For calculation of the apparent degree of binding, the ratio total/free is taken as equal to the ratio C_i/C_o. Actually, as a result of a Donnan effect, the free concentration on the polymer-containing side of the membrane is less than C_o and the actual ratio total/free $> C_i/C_o$. The complexity of the systems prevents calculation of the Donnan correction for most of the examples of binding considered in this report but, if applied, such a correction would indicate even greater binding than the apparent ratios reported here.

RESULTS

Tetracaine Hydrochloride and Procaine Hydrochloride.—The relative affinity of tetracaine hydrochloride and procaine hydrochloride for polysorbate 80 is shown in Fig. 1. At a concentration of 5% polysorbate 80, approximately 20% of the total tetracaine is bound to the surfactant; at a concentration of 10% polysorbate 80, approximately 30% of the tetracaine is bound. The procaine ion does not appear to interact significantly with the polysorbate under the conditions of this study.

Chlorpromazine Hydrochloride.—Figure 2 illustrates interaction of chlorpromazine hydrochloride with polysorbate 80 in aqueous solution. The addition of electrolyte appears to enhance the interaction considerably. In an aqueous solution of 2% polysorbate 80, approximately 50% of the total chlorpromazine present is bound to the surfactant; when 0.1% $NaHSO_3$ is present in this system (to retard oxidation), approximately 75% of the chlorpromazine is bound; when the system consists of 2% polysorbate 80, 0.1% $NaHSO_3$, and 0.5 *M* KCl, approximately 90% of the chlorpromazine is bound to the surfactant.

Promethazine Hydrochloride.—The interaction of promethazine hydrochloride with polysorbate 80 is illustrated in Fig. 3. At a concentration of 2% polysorbate 80 and in the presence of 0.1% $NaHSO_3$ (to retard oxidation of the promethazine), approximately 38% of the total promethazine is bound to the surfactant; when KCl is added to this system to establish a total ionic strength of approximately 0.6, approximately 70% of the promethazine is bound to the polysorbate 80.

Dodecylpyridinium Chloride and Cetylpyridinium Chloride.—DeLuca and Kostenbauder previously presented data to illustrate the high degree of

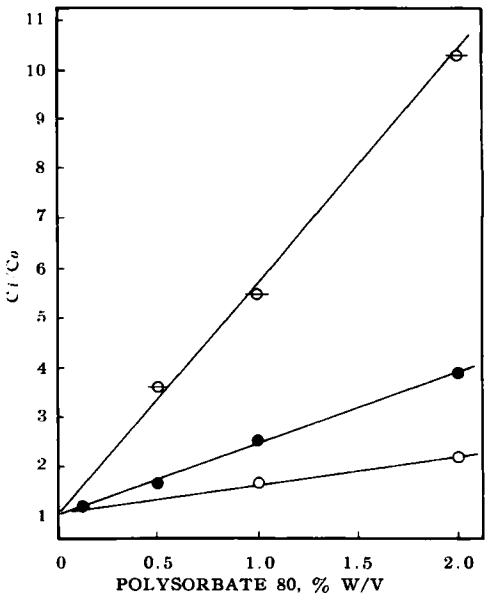

Fig. 2.—Influence of electrolyte on binding of chloropromazine hydrochloride by polysorbate 80 at 30°. Sodium bisulfite added as antoxidant. Key: O, in polysorbate 80; ●, in polysorbate 80 plus 0.1% $NaHSO_3$; ⊖, in polysorbate 80 plus 0.1% $NaHSO_3$ and 0.5*M* KCl.

interaction between cetylpyridinium chloride and polysorbate 80 (16). These data are reproduced in Fig. 4 to permit comparison with the interaction between dodecylpyridinium ion and polysorbate 80. At a concentration of 2% polysorbate 80 approximately 40% of the dodecylpyridinium ion is bound to the polysorbate; while at this concentration of polysorbate 80, approximately 97% of the cetylpyridinium ion is bound.

Methylrosaniline Chloride.—Figure 5 illustrates the interaction of the cationic dye methylrosaniline chloride with polysorbate 80. These adsorption isotherms indicate that the nature of the binding may change markedly with concentration of free methylrosaniline ion, and it is not possible to apply the relatively simple description of the binding used previously; *i.e.*, it is not sufficient to describe the binding in terms of a definite percentage of the methylrosaniline chloride bound to the polysorbate at any specific polysorbate concentration. For example, at a concentration of 0.25% polysorbate 80, the percentage of dye bound to the surfactant varied from approximately 70% at the lower dye concentrations to 40% at the higher dye concentrations. At a concentration of 5% polysorbate 80, approximately 90% of the dye was bound throughout the concentration range studied.

Sodium Naphthalene Sulfonate.—Sodium naphthalene sulfonate was included in this study to demonstrate that anionic as well as cationic agents might be bound to nonionic surface-active agents. The interaction of the naphthalene sulfonate anion with polysorbate 80 is illustrated in Fig. 6. In the presence of a 2% aqueous solution of polysorbate 80, approximately 7% of the total naphthalene sulfonate ion is bound to the polysorbate; however, when 0.1 *M* KCl was added to this system, approximately 17% of the naphthalene sulfonate was bound to the polysorbate. The naphthalene sulfonate ion was selected for study because of the absence of functional groups, other than sulfonate, which might permit hydrogen bonding to the polyether portion of the polysorbate, and because this ion is essentially in the completely dissociated form in aqueous solution, thereby eliminating the possibility of an interaction being attributed to binding of undissociated molecules to the polysorbate.

Methyl Orange.—The interaction of the anionic dye methyl orange with polysorbate 80 is shown in Fig. 7. As with methylrosaniline chloride, the fraction of dye bound to the surfactant at any polysorbate concentration changes markedly with the concentration of unbound dye; in the presence of 5% polysorbate 80, the percentage of bound dye ranged from 96% at low dye concentrations to 83% at higher dye concentrations.

Drug Ions Not Bound to Polysorbate 80.—No significant interaction was observed for the following drug ions in the presence of aqueous solutions of polysorbate 80: ephedrine hydrochloride, sulfathiazole sodium, methapyrilene hydrochloride, and diphenhydramine hydrochloride.

DISCUSSION

Nature of Interaction with Polysorbate 80.—The interaction of drug ions with polysorbate 80 appears to be similar to the interaction of cetylpyridinium ion with polysorbate 80 as described by DeLuca and Kostenbauder (16). It appears that ions having a large hydrophobic group can undergo association with the polysorbate micelle to form a type of mixed-micelle. Binding of ions such as dodecylpyridinium and naphthalene sulfonate, which exist entirely in dissociated form in aqueous solution and have no other functional groups which might be expected to hydrogen bond to the ethylene oxide portion of the polysorbate, indicates that the necessary structural feature for an organic ion to interact with a nonionic surface-active agent is that of a relatively large hydrophobic portion.

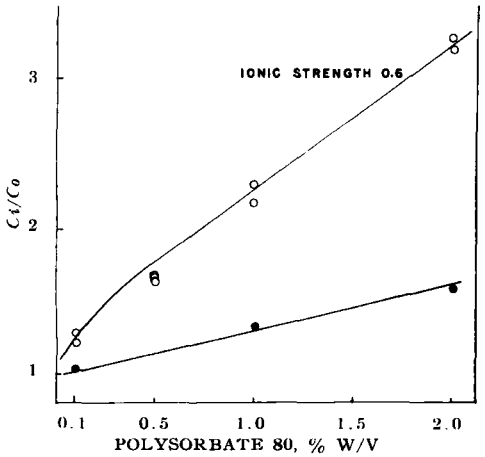

Fig. 3.—Binding of promethazine hydrochloride by polysorbate 80 at 30°. Solid symbols correspond to systems containing 0.1% $NaHSO_3$ as antoxidant; open symbols represent systems containing 0.1% $NaHSO_3$ plus sufficient KCl to produce an ionic strength of 0.6.

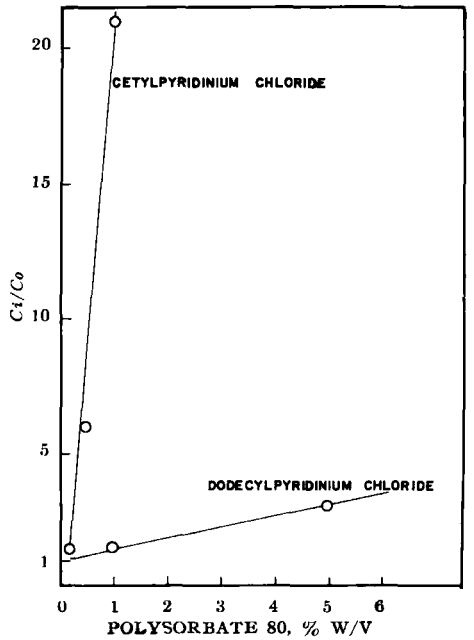

Fig. 4.—Relative binding by polysorbate 80 of dodecylpyridinium chloride and cetylpyridinium chloride at 30°. Binding data for cetylpyridinium chloride reproduced from DeLuca and Kostenbauder (16).

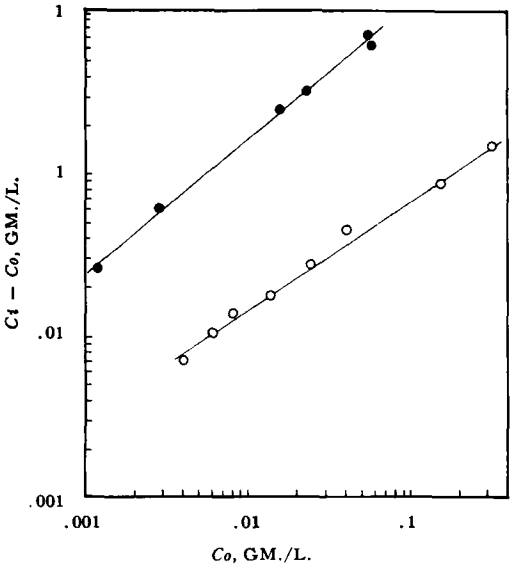

Fig. 5.—Binding of methylrosaniline chloride by polysorbate 80 at 30°. Open symbols represent solutions containing 0.25% polysorbate 80; solid symbols represent 5% polysorbate 80.

Ions such as ephedrine, sulfathiazole, methapyrilene, diphenhydramine, and procaine apparently are not sufficiently hydrophobic to associate with polysorbate 80 micelles. The presence of the *n*-butyl substituent on the *p*-amino group of tetracaine apparently confers sufficient hydrophobic character for this ion to associate with the polysorbate. The importance of the hydrophobic character of the ion is further illustrated in the comparative binding of cetylpyridinium ion and dodecylpyridinium ion.

Salt Effects.—For a weakly bound electrolyte such as sodium naphthalene sulfonate, enhanced binding to polysorbate 80 in the presence of added electrolyte might be accounted for on the basis of swamping of a Donnan effect. Adsorption of hydrophobic ions would, in effect, cause the polysorbate 80 to act as an ionic polymer and necessitate a Donnan correction for the binding studies. If the adsorbed hydrophobic ion binds few counter ions, and if it is assumed that the hydrophobic ion under study does not form micelles at the concentrations considered, then a Donnan correction can be applied in the following manner

Let

C_o = concentration of hydrophobic ion in the outside or nonpolymer containing compartment.

C_i = concentration of total hydrophobic ion in the inside compartment and consists of bound plus free.

$C_{i,\,free}$ = concentration of free hydrophobic ion in inside compartment.

Donnan equilibrium:

$$(C_o)^2 = C_i \times C_{i,\,free}$$

$C_{i,\,free}$ is therefore less than C_o and actual ratios of total/free drug are greater than the observed ratio C_i/C_o.

This Donnan correction was applied to the naphthalene sulfonate–polysorbate 80 data. As

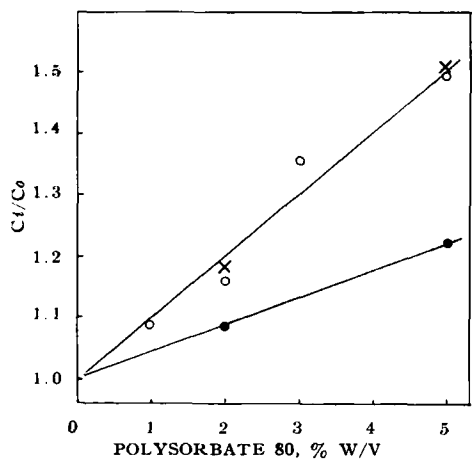

Fig. 6.—Binding of sodium naphthalene sulfonate by polysorbate 80 at 30°. Key: O, with 0.1 *M* KCl; ●, without KCl; ×, points obtained by applying a Donnan correction to the binding data obtained in absence of KCl.

shown in Fig. 7, the binding data in absence of salt, when corrected for the Donnan effect, are in excellent agreement with the degree of binding observed in the presence of 0.1 *M* swamping electrolyte. The effect of electrolyte on the apparent degree of binding of naphthalene sulfonate to polysorbate 80 can therefore be attributed almost entirely to elimination of the Donnan effect.

Other systems studied are not amenable to correction for this Donnan effect. Where there is a high degree of interaction with the polysorbate 80, it is almost a certainty that there is considerable counter-ion retention, just as there would be in micelles of ionic surfactants. While it is not feasible to apply a Donnan correction, it can be predicted that any correction for a Donnan effect could only lead to an even greater degree of interaction than that indicated by the ratio C_i/C_o; therefore, total/free $\geqq C_i/C_o$ in all cases.

Where there is considerable counter-ion retention by bound hydrophobic ions, added electrolyte may have a further effect as indicated by application of the law of mass action to micelle formation. Where A^+ represents the hydrophobic ion and B^- the counter-ion, the interaction with polysorbate may be represented as

$$aA^+ + bB^- + Tw \rightleftarrows (A_a^+ B_b^- \, Tw) \qquad a > b$$

$$K = \frac{(A_a^+ B_b^- \, Tw)}{(A^+)^a \, (B^-)^b \, Tw}$$

The concentration of bound hydrophobic ion, (A^+), is

$$\frac{K \, (A^+)^a \, (B^-)^b \, (Tw)}{a}$$

and an increase in the concentration of the counter-ion (B^-), on addition of an electrolyte such as KCl, would be expected to increase markedly the binding of the hydrophobic ion, A^+.

For a strongly bound electrolyte such as chlorpromazine hydrochloride, the effect of added electrolyte on the observed ratio C_i/C_o might therefore be attributed in part to swamping of the Donnan

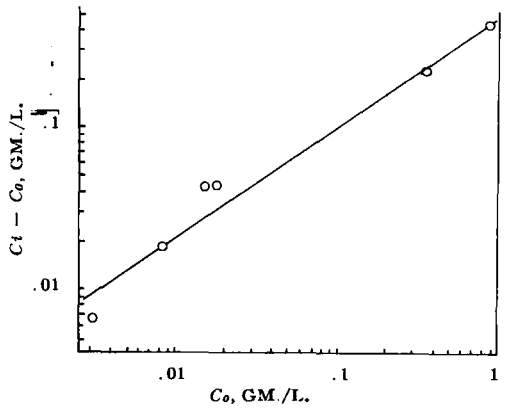

Fig. 7.—Binding of methyl orange by 5% polysorbate 80 at 30°.

effect and in part to the effect of increasing concentration of counter-ion on the equilibrium as predicted by the law of mass action.

Pharmaceutical Significance of These Interactions.—Perhaps the most obvious consequence of interactions such as those described in this paper is the influence of the binding on the release of a drug from a formulation containing a nonionic surfactant or the availability or activity of a germicidal agent in such a formulation. Interactions in which a large percentage of the drug is bound to a component in the formulation would be expected to modify greatly the release of the drug and subsequent absorption or access to the site of action. This might be particularly true in topical formulations where nonionic surface-active agents may be employed in concentrations of 5% or more in the aqueous phase. Of course absorption of drugs or release of drugs from formulations is an extremely complex process, and binding of drug to surfactant is only one aspect of the problem; it may well be that other factors to be considered make it highly desirable that the surfactant be employed in a large concentration in spite of possible interactions with the drug. Nevertheless, it is important to recognize that nonionic surfactants can interact with ionic drugs and that this interaction might be expected to exert considerable influence on the release of some drug ions from such systems.

The very pronounced effect of interaction between nonionic surfactants and quaternary ammonium compounds on the availability and antimicrobial activity of the quaternary ammonium compounds has been demonstrated previously (16). Such interactions between nonionic agents and quaternary ammonium compounds, or other materials having antimicrobial activity, are not always undesirable; the inactivation of quaternary ammonium compounds by addition of a nonionic agent is a common procedure in carrying out sterility tests where there normally would be interference from the antimicrobial agent present in the formulation.

A second important consequence of drug-surfactant interactions is the effect of such interaction on the stability of the drug. Although no data can be cited for systems discussed in this paper, Riegelman (18) has demonstrated the very significant effects on drug stability which might arise from association of drugs with surfactant micelles. Riegelman (18), Nogami, *et al.* (19, 20), and Nakajima (21) have shown that a number of drugs with labile ester linkages can be significantly stabilized against ester hydrolysis when solubilized in aqueous solutions of micellar nonionic surface-active agents. On the other hand, Motsavage and Kostenbauder (22) have recently demonstrated that binding of alkyl sulfates to nonionic micelle-forming agents may increase the rate of the hydronium ion catalyzed hydrolysis of the sulfate ester as much as 60-fold. Whether a drug is more or less stable when associated with the nonionic surfactant micelle will depend on the positioning of the labile group in or on the micelle and on the nature of the decomposition to which it is subject.

SUMMARY

Data have been presented to show that drug ions can interact in aqueous solution with a nonionic surface-active agent to an extent which might markedly influence the stability of the drug or the release of the drug from a formulation.

Only those drug ions having a relatively large hydrophobic group were found to interact with polysorbate 80. The mechanism of the interaction appears to be similar to the mixed micelle formation which occurs on interaction of cetylpyridinium ion with polysorbate 80. Addition of electrolyte appears to enhance the interaction considerably.

The following ions were found to bind to polysorbate 80 in aqueous solution: cetylpyridinium, methylrosaniline, methyl orange, chlorpromazine, dodecylpyridinium, promethazine, tetracaine, and naphthalene sulfonate.

The following ions did not appear to interact with polysorbate 80 to a significant extent: ephedrine, sulfathiazole, diphenhydramine, methapyriline, and procaine.

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Thiaindanones

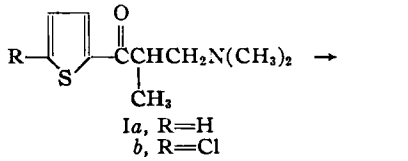
Thiophene Isosteres of Indanone

By JOSEPH SAM and ALONZO C. THOMPSON

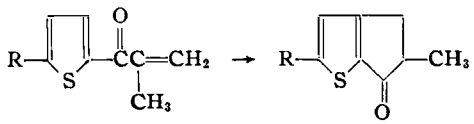
The preparation of substituted 4- and 6-thiaindanones[1] (thiophene isosteres of indanone) *via* intramolecular acylation of β-(2- and 3-thienyl)propanoic acids is described. The Wolff-Kishner and sodium borohydride reductions provided the corresponding thiaindan and thiaindanol (thiophene isosteres of indan and indanol), respectively.

THE IMPORTANCE of molecular size and shape in determining biological properties has been recognized for many years. The chemical, physical, and biological properties of isosteres have been studied extensively (2), since they possess steric similarities. Interest in the synthesis and in the biological properties of isosteres is kindled in the hope that useful medicinal agents and essential clues to the mechanism of drug action may be obtained.

Burckhalter and Sam (1) prepared the thiophene isostere (III*a*) of 2-methyl-1-indanone (IV) but did not report biological activity. Since the thiaindanones (III) represented a ring system that had not been investigated extensively, we were interested in the preparation of other thiophene isosteres of indanone and in ascertaining their biological properties. Moreover, since the presence of the sulfur atom gives rise to an unsymmetrical molecule, we were also interested in the preparation of the isomeric thiophene isosteres (V). During the course of our work Poirier (3) described the preparation of some closely related derivatives of this ring system.

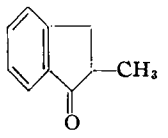


I*a*, R=H
b, R=Cl



II*a*, R=H
b, R=Cl

III*a*, R=H
b, R=Cl



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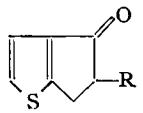
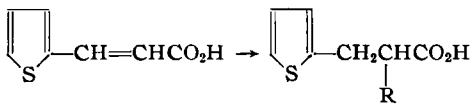
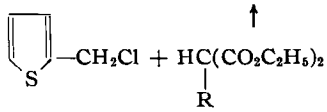


V

The method (I→III) reported by Burckhalter and Sam (1) for the preparation of III*a* is not generally applicable to other substituted thiaindanones. Except for the preparation of the chloro derivative (III*b*) we made use of the intramolecular acylation of substituted β-thienylpropanoic acids. The substituted β-(2-thienyl) propanoic acids (VII) were prepared by the condensation of 2-thenyl chloride with substituted malonates according to the procedure described by Levy (4) and modified by Sam and Plampin (5) for the preparation of substituted hydrocinnamic acids. The unsubstituted acid (VII, R=H) was obtained from the reduction of the corresponding acrylic acid (VI)



VI VII



The reduction of VI was accomplished either with 10% sodium-lead alloy in water at room temperature or catalytically at 3–4 Atm. with 10% palladium-on-charcoal catalyst. Contrary to expected results, we obtained a good yield in the catalytic reduction of VI to VII (R=H).

The substituted β-(3-thienyl)propanoic acids (VIII) were prepared from 3-thenyl bromide *via* condensation with substituted malonates (6)

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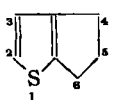
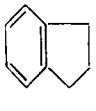
Presented to the Scientific Section, A.PH.A., Miami meeting, May 1963.

[1] Because of the cumbersome chemical name originally assigned (1) to III*a*, *i.e.*, 4,5-dihydro-5-methyl-6H-cyclopenta-(b)thiophen-6-one, we propose the name thiaindan for the thiophene isostere (i) of indan (ii), and the names thiaindanone and thiaindanol for the corresponding isosteres of indanone and indanol, respectively.

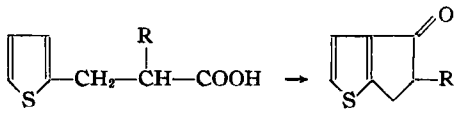


i



ii

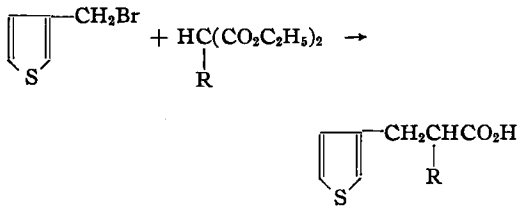
TABLE I.—THIAINDAN-4-ONES



VII V

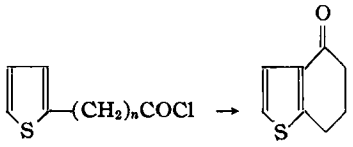
R	Solvent for VII	Reaction Temp., °C.	Reaction Time, hr.	Yield, %	Cyclization Agent
CH_3	C_6H_5Cl	130	0.25	54	PPA[a]
CH_3	C_6H_5Cl	120	0.25	48	PPA
CH_3	...	120	0.25	36	PPA
CH_3	C_6H_5Cl	115	0.25	37	PPA
CH_3	...	30–80[b]	0.25–12[b]	0	PPA
CH_3[c]	CS_2	...	0.5	0	$AlCl_3$
CH_3[c]	CS_2	...	2	0	$AlCl_3$
CH_3[c]	CS_2	...	2	0	$SnCl_4$
CH_3	...	30, 45	0.75	0	H_2SO_4
CH_3	...	30	12	0	HF
H	CH_2Cl_2	120	0.25	36	PPA
C_6H_5	C_6H_5Cl	115	0.25	16	PPA
H	...	30	12	0	HF
C_6H_5	...	30	12	0	HF

[a] Polyphosphoric acid. [b] Eight separate experiments varying the temperature and duration. [c] Acid chloride.



VIII

Intramolecular acylation studies of the substituted β-(2 and 3-thienyl)propanoic acids are summarized in Tables I and II. Our studies (Table I) with α-methyl-β-(2-thienyl)propanoic acid revealed that a solution of the organic acid in chlorobenzene when added to polyphosphoric acid (PPA) at an elevated temperature (preferably 130°) over a short period of time gave the best results. The use of other dehydrating agents such as sulfuric acid and liquid hydrogen fluoride gave negligible results. In contrast to the good results obtained by Fieser and Kennelly (7) with the cyclization of γ-(2-thienyl)butanoyl chloride (IX, $n=3$) to X, we were unable to cyclize β-(2-thienyl)propanoyl chloride (IX, $n=2$) to V (R=H) utilizing the same conditions. Aluminum chloride also was ineffective in the cyclization of the acid chloride

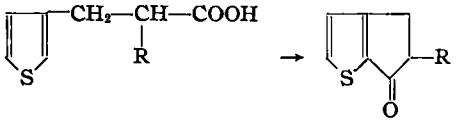


IX X

The intramolecular acylation of substituted β-(3-thienyl)propanoic acids (VIII) to the corresponding substituted 6-thiaindanones (XI, Table II) was accomplished without difficulty and in moderate yields with either PPA or liquid hydrogen fluoride. In contrast to the sluggish cyclization of the isomeric 2-thienyl acids (VII), the ease with which this cyclization was accomplished is explained on the basis of the differences in the reactivity of the 2 and 3 positions of thiophene.

The 5-methyl-6-thiaindanone, III*a*, undergoes

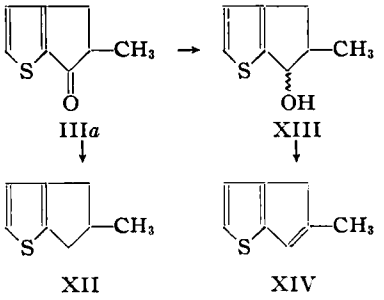
TABLE II.—THIAINDAN-6-ONES



VIII XI

R	Solvent for VIII	Reaction Temp., °C.	Reaction Time	Yield, %	Cyclization Agent
H	C_6H_5Cl	75	5 min.	10.4	PPA
H	...	30	12 hr.	43.9	HF
ϕ	C_6H_5Cl	130	15 min.	21.7	PPA
ϕ	...	30	48 hr.	71.9	HF
CH_3	C_6H_5Cl	70	1 hr.	60.0	PPA
CH_3	...	70	1 hr.	0.0	PPA

the typical Wolff-Kishner reduction (8) to give the thiophene isostere (XII) of 2-methylindan; sodium borohydride reduction (9) of III*a* yielded a thiophene isostere (XIII) of 2-methyl-1-indanol. Preliminary attempts to obtain the isomeric alcohol have been unsuccessful. Since analogous reductions of 2-methyl-1-indanone to 2-methyl-1-indanol with sodium borohydride have provided exclusively the *cis*-2-methyl-1-indanol, we believe the reduction of III*a* likewise gives the *cis* isomer.



The dehydration of 5-methyl-6-thiaindanol occurred on heating and resulted in a product (XIV?) which gave the characteristic tests for unsaturation. However, this material rapidly deteriorated and was not fully characterized.

Preliminary pharmacological studies in mice demonstrated that the semicarbazones of III*a* and V (R = CH_3) exhibit weak anticonvulsant activity. The acid, VIII (R = CH_3), and the ketone, XI (R = H), also exhibited weak anticonvulsant activity. The semicarbazone of III*a* produced a weak antihypertensive response in both rats and dogs.

EXPERIMENTAL[2]

β-(2-Thienyl)propanoic Acid.—*Method A.*—A 9.6% lead-sodium alloy (100 Gm.) was added to a stirred solution of 15.4 Gm. (0.1 mole) of β-(2-thienyl)acrylic acid in 200 ml. of 10% sodium hydroxide solution over a period of 2 hours. The mixture was filtered and the basic filtrate neutralized with concentrated hydrochloric acid. The precipitate was removed by filtration, air dried, and recrystallized from petroleum ether (b.p. 30–60°) to give 10 Gm. (64%) of product, m.p. 48–49° (lit. (10) m.p. 46–48°).

Method B.—A solution of 15.4 Gm. (0.1 mole) of β-(2-thienyl)acrylic acid in 200 ml. of methanol was treated with 2 Gm. of 10% palladium-carbon catalyst and hydrogenated at 42 p.s.i. Hydrogenation was complete at the end of 1 hour; no further hydrogen uptake occurred during an additional hour. The catalyst was removed by filtration and the solvent was distilled *in vacuo* leaving 12.6 Gm. (80%) of product, m.p. 48–49°. The infrared spectra of samples from *Methods A* and *B* were identical. Mixed melting points showed no depression.

[2] All melting points were taken on a Fisher-Johns apparatus and are corrected; boiling points are uncorrected. Infrared spectra were determined on a Perkin-Elmer model 137 infracord spectrophotometer.

α-(Methyl-β-(3-thienyl)propanoic Acid.—To a solution of 234.6 Gm. (1.4 moles) of diethyl methylmalonate in ethanolic sodium ethoxide (made from 29.7 Gm. (1.3 Gm. At.) of sodium and 600 ml. of ethanol) was added through a dropping funnel 230 Gm. (1.3 moles) of 3-thenyl bromide and the mixture heated at reflux for 7 hours. A solution of 300 Gm. (5.36 moles) of potassium hydroxide in 300 ml. of water was added to the ethanol solution and the mixture refluxed for 24 hours. The mixture was concentrated *in vacuo* to about 400 ml. The residual mixture was treated with 500 ml. of water, cooled in an ice bath, neutralized with concentrated hydrochloric acid, and extracted with ether. Evaporation of the ether and distillation of the residual oil gave 151 Gm. of product (68%), b.p. 141–145° (5 mm.) n_D^{28} 1.5225.

Anal.—Calcd. for $C_8H_{10}O_2S$: C, 56.5; H, 5.9. Found: C, 56.4; H, 6.2.

The amide was prepared in the usual manner and recrystallized from an ethanol-water mixture and water, respectively, m.p. 121–122°.

Anal.—Calcd. for $C_8H_{11}NOS$: C, 56.8; H, 6.5. Found: C, 57.0; H, 6.8.

α-Phenyl-β-(3-thienyl)propanoic Acid.—The procedure for the synthesis of α-methyl-β-(3-thienyl)-propanoic acid was employed using 177 Gm. (1.0 mole) of 3-thenyl bromide and 238 Gm. (1.0 mole) of diethyl phenylmalonate. Ninety-seven grams (42%) of product were obtained, b.p. 194° (2.5 mm.); m.p. 87–89°.

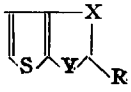
The amide was prepared in the usual manner and recrystallized from an ethanol-water mixture, m.p. 130–132°.

Anal.—Calcd. for $C_{13}H_{13}NOS$: C, 67.5; H, 5.6; N, 6.1. Found: C, 67.6; H, 5.5; N, 6.0.

Cyclization of β-(2- and 3-Thienyl)propanoic Acids to Thiaindanones (Tables I, II). *Polyphosphoric Acid Method.*—The appropriate β-(2- and 3-thienyl)propanoic acid *per se* or dissolved in 100 ml. of solvent was added with stirring to 200 Gm. of polyphosphoric acid, preheated to the desired temperature. The dry acid was added essentially in one lot, whereas the solution of the acid in the solvent was added over a 15-minute period. Thereafter, the mixture was poured with stirring into 200 ml. of ice water. The mixture was extracted with either ethyl acetate or ether. The combined extracts were washed with water, 10% sodium bicarbonate solution, and dried over anhydrous sodium sulfate. Evaporation of the solvent and distillation or recrystallization of the residue provided the desired product (Table III). Infrared spectra in carbon tetrachloride showed strong carbonyl absorption at 1625–1740 $cm.^{-1}$

Hydrogen Fluoride Method.—The procedure was adapted from that of Heinzelmann and co-workers (11) for the cyclization of substituted phenylpropanoic acids. A 250-ml. copper retort containing 0.1 mole of the appropriate thienylpropanoic acid per 150 Gm. of liquid hydrogen fluoride was allowed to remain overnight in a fume hood. The contents of the retort were poured into a copper beaker and the remaining hydrogen fluoride evaporated. An ethereal solution of the residual material was washed with water and 10% sodium bicarbonate solution, respectively. The product was isolated as described above.

TABLE III.



R	X	Y	M.p., °C.	B.p., °C.	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found
H	CO	CH_2	115–117	110 (0.2 mm.)	C_7H_6OS	60.9	61.4	4.4	4.6	...	...
CH_3	CO	CH_2	39–40	88–98 (0.7 mm.)	C_8H_8NOS	...	...	...	...	...	...
CH_3	CO	CH_2	243–245[a]	...	$C_{14}H_{12}N_4O_4S$[b]	50.6	50.8	3.6	3.6	16.9	16.7
CH_3	CO	CH_2	121–122[c]	...	C_8H_9NOS[d,e]	57.5	57.7	5.4	5.3	8.4	8.3
CH_3	CO	CH_2	190–192[c]	...	$C_9H_{11}N_3OS$[f]	51.7	51.7	5.3	5.1	...	...
C_6H_5	CO	CH_2	103–104	185 (2 mm.)	$C_{13}H_{10}OS$	72.9	73.1	4.7	4.9	...	...
H	CH_2	CO	90–91	110 (7 mm.)	C_7H_6OS	60.0	61.1	4.3	4.4	...	...
CH_3	CH_2	CO	...	106 (2 mm.)	C_8H_8NOS[g]	...	...	...	...	...	...
CH_3	CH_2	CO	183–185[c]	...	$C_9H_{11}NO_3S$[f]	51.7	51.8	5.3	5.4	...	...
CH_3	CH_2	CO	121–123[c]	...	C_8H_9NOS[d]	57.5	57.4	5.4	5.8	...	...
C_6H_5	CH_2	CO	96–97[c]	180 (1 mm.)	$C_{13}H_{10}OS$[h]	72.9	73.0	4.6	4.7	...	...
CH_3	CH_2	CO	...	91 (0.8 mm.)	C_8H_8ClNOS[i]	...	...	...	...	...	...
CH_3	CH_2	CO	218–219[a]	...	$C_{14}H_{11}ClN_4O_4S$[b,i]	45.8	45.5	3.0	2.8	15.3	15.2
CH_3	CH_2	CH_2	...	69–73 (17 mm.)	$C_8H_{10}S$	69.6	69.4	7.3	7.4	...	...
CH_3	CH_2	CHOH	51	114 (6 mm.)	$C_8H_{10}OS$	62.3	62.5	6.2	6.5	...	...

[a] Recrystallized from an ethanol-ethyl acetate mixtures. [b] 2,4-Dinitrophenylhydrazone. [c] Recrystallized from ethanol. [d] Oxime. [e] Calcd. for S, 18.6. Found: S, 18.9. [f] Semicarbazone. [g] Reference 2. [h] Calcd. for S, 15.0. Found: S, 15.1. [i] 2-Chloro derivative.

5-Chloro-2-(α-methylacrylo)thiophene.—The procedure described by Burckhalter and Sam (1) for the preparation of 2-(α-methylacrylo)thiophene was followed. From 145 Gm. (0.83 mole) of 5-chloro-2-propionylthiophene, 66.5 Gm. (0.83 mole) of dimethylamine hydrochloride, 24.9 Gm. (0.83 mole) of formaldehyde, there was obtained 49 Gm. (26%) of product, b.p. 130° (6 mm.); n_D^{25} 1.5576.

Anal.—Calcd. for C_8H_7ClOS: C, 51.5; H, 3.8. Found: C, 51.7; H, 4.0.

2-Chloro-5-methylthiaindan-6-one.—The procedure described by Burckhalter and Sam (1) for the preparation of 5-methylthiaindan-6-one was followed. From 48.7 Gm. (0.26 mole) of 5-chloro-2-(α-methylacrylo)thiophene there was obtained 20 Gm. (50%) of product, b.p. 91° (0.8 mm.); n_D^{25} 1.5887.

The 2,4-dinitrophenylhydrazone was prepared in the usual manner and recrystallized eight times from an ethanol–ethyl acetate mixture, m.p. 218–219°.

5-Methylthiaindan.—In a 500-ml. three-necked flask were placed 45.6 Gm. (0.3 mole) of 5-methylthiaindan-6-one, 30 ml. of 85% hydrazine hydrate, 40 Gm. (0.71 mole) of potassium hydroxide, and 300 ml. of diethylene glycol. The general method (7) for the Wolff-Kishner reaction was followed. The flask was heated on a steam bath until most of the potassium hydroxide had dissolved, then heated at reflux for 1 hour. The solution was partially distilled until the internal temperature of the flask reached 200°. The resulting solution was heated at 200° for 3 hours, cooled to 90°, and treated with 100 ml. of water. The reaction mixture was distilled until the internal temperature reached 200°. The oil from the aqueous distillate was separated, dried over calcium chloride, and distilled. Distillation was difficult because of foaming under reduced pressure. After distillation, 20 Gm. (50%) of product was obtained, b.p. 69–73° (17 mm.); n_D^{25} 1.5306. The infrared spectrum did not show carbonyl absorption between 1600 and 1700 cm.$^{-1}$

5-Methylthiaindan-6-ol.—In a 500-ml. three-necked flask fitted with a condenser, magnetic stirrer, and a dropping funnel a solution of 40 Gm. (0.265 mole) of 5-methylthiaindan-6-one in 200 ml. of methanol was placed. Through the dropping funnel 7.6 Gm. (0.2 mole) of sodium borohydride in 100 ml. of methanol was added with stirring. The addition of the sodium borohydride solution was regulated to keep the solution refluxing. After the addition of sodium borohydride, the reaction mixture was refluxed for 1 hour. The methanol was distilled *in vacuo;* the white solid was treated with 165 ml. of 20% sodium hydroxide solution and extracted three times with 100-ml. portions of ether. The combined ether extracts were dried over anhydrous magnesium sulfate. The ethereal solution was distilled to give 30 Gm. (75%) of product, b.p. 114° (6 mm.), m.p. 51–52°. The infrared spectrum in carbon tetrachloride showed characteristic hydroxyl absorption at 3550 cm.$^{-1}$, whereas carbonyl absorption was absent.

cis-2-Methyl-1-indanol.—The above procedure was followed using 12 Gm. (0.08 mole) of 2-methyl-1-indanone, 3.5 Gm. (0.1 mole) of sodium borohydride, and 200 ml. of methanol. Twelve grams (100%) of *cis*-2-methyl-1-indanol was obtained, m.p. 43–46° (lit. (12) m.p. 50–51°).

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Synthesis of Amides of Diphenic Acid as Potential Antispasmodic Agents

By GLENN L. JENKINS, CHARLES S. DAVIS, ADELBERT M. KNEVEL, and DAVID S. YODER†

Twenty-six new amides of diphenic acid have been synthesized as potential antispasmodics. All of the described compounds were screened for pharmacological activity; none of the new structures demonstrated significant antispasmodic activity to warrant further investigation. Details of the pharmacological screening will be reported by G. K. W. Yim in another paper.

It is well documented that many compounds containing the ester group have demonstrated pharmacological activity as antispasmodics and local anesthetics. Since the amide group is quite similar to the ester both chemically and pharmacologically, it might be expected that the amides of diphenic acid would produce "active" compounds (1, 2). In some instances in which the ester group has been substituted by an amide group (as in the case of procainamide) the pharmacological action has been prolonged and the toxicity reduced. The prolonged activity has been attributed to the slow rate of hydrolysis of the amide as compared to the ester (3).

Diphenic acid as an inert nontoxic substance (4) has properties which may make it desirable as a carrier of pharmacologically active groups. Demers and Jenkins (5) prepared a number of derivatives of diphenic acid which were found to possess spasmolytic activity about equal to that of atropine (6).

EXPERIMENTAL

Diphenic acid was prepared by the method of Atkinson and Lawler (7) with the modification of Demers and Jenkins (5). Diphenic anhydride was prepared by the general procedure of Roberts and Johnson (8). Diphenic acid, diphenimide, and the monoethyl ester of diphenic acid were prepared by the methods of Underwood and Kochmann (9). Potassium diphenimide was prepared by reacting equimolar quantities of potassium ethoxide in anhydrous ethanol with diphenimide; ethyl diphenoyl chloride by the procedure described by Demers and Jenkins (5). The amines used for this investigation were supplied through the courtesy of Union Carbide and Carbon; β-diethylaminoethyl chloride HCl and β-dimethylaminoethyl chloride HCl were supplied through the generosity of the Michigan Chemical Corp. The dialkylaminoalkyl chlorides not commercially available were synthesized by the method of Mason and Block (10) and according to Adams and Whitmore (11). Liberation of the "free" chloro-base compounds from the hydrochloride salts was accomplished by the procedure of Burtner (12).

It is interesting to note that in the attempted preparations of diphenimide derivatives the imide ring opened to produce an ester and an amide. The product formed was identical to that prepared from the ester-acid chloride reaction.

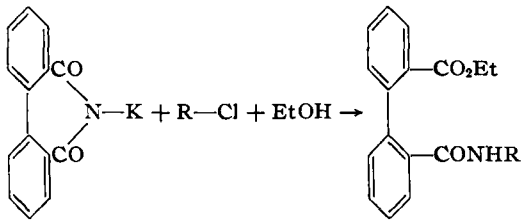

Table I lists the amide derivatives synthesized by one of the following procedures.

Procedure A

Ethyl N-(Dialkylaminoalkyl)-diphenamate.—To 100 ml. of anhydrous ethyl alcohol was added 43 m*M* of metallic potassium with cooling. A 43-m*M* quantity of diphenimide was added to the potassium ethoxide solution. After the diphenimide had dissolved, 43 m*M* of the appropriate dialkylaminoalkyl chloride was added and the mixture was refluxed for 12–14 hours. At the conclusion of the reflux period, a white precipitate formed which was removed by filtration. The filtrate was concentrated under vacuum and the residue taken up in anhydrous ether. Dry hydrogen chloride was passed into the etheral extract; a white precipitate formed, which crystallized upon standing. The crystals were collected, dried, and recrystallized.

Procedure B

Ethyl N-(Dialkylaminoalkyl)-diphenamate.—To 25 m*M* of ethyl diphenoyl chloride in 20 ml. of dry benzene was added slowly 25 m*M* of the appropriate dialkylaminoalkylamine in 10 ml. of dry benzene with stirring and cooling in an ice bath. After completion of the reaction, the mixture was cooled overnight in a refrigerator. If the product did not crystallize, the syrupy residue was treated with fresh portions of cold dry ether until a precipitate was formed. The crystals were collected, dried, and recrystallized.

Procedure C

Ethyl N-Alkyldiphenamate.—A 25-m*M* quantity of ethyl diphenoyl chloride was added with stirring and cooling to 50 m*M* of alkylamine in 10 ml. of dry benzene or chloroform. After the mixture was allowed to stand overnight in a refrigerator, the

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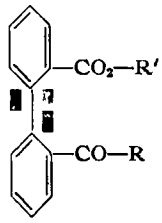
TABLE I.—AMIDES OF DIPHENIC ACID



R	R′	Yield, %	M.p., °C.[a]	Nitrogen, % Calcd.	Nitrogen, % Found	Method of Synthesis
Diethylaminoethylamino . HCl	Ethyl	60	161–162	6.91	6.96	A
Dimethylaminoethylamino . HCl	Ethyl	74	159–160	7.45	7.33	A
Pyrrolidinoethylamino . HCl	Ethyl	43	120–121	6.95	6.97	A
Piperidinoethylamino . HCl	Ethyl	46	156–157	6.73	7.02	A
Morpholinoethylamino . HCl	Ethyl	40	147–149	6.70	6.95	A
Diethylaminopropylamino . HCl	Ethyl	51	140–141	6.70	6.85	A and B
Piperidinopropylamino . HCl	Ethyl	47	131–132	6.50	6.39	A
Dimethylaminopropylamino . HCl	Ethyl	60	124–126	7.17	7.42	B
Di-*n*-butylamino	Ethyl	70	51–52	3.67	3.60	C
Di-*n*-butylamino	H	57	140–142	3.79	3.59	E
2-Hydroxyethylamino	Ethyl	67	88–89	4.47	4.30	C
N,N-di-(2-hydroxyethyl)amino	Ethyl	54	84–85	3.92	3.93	C
2-Hydroxypropylamino	Ethyl	80	93–95	4.28	4.30	C
N,N-(Di-isobutyl)amino	H	38	206–208	3.79	3.59	E
N,N-(Di-isopropyl)amino	H	21	185–186	4.10	3.95	E
N,N-Diphenylamino	Ethyl	55	136–137	3.32	3.40	D
Morpholino	H	81	250–251	4.50	4.37	E
Piperidino	H	75	157–159	4.52	4.40	E
2-Methylpiperidino	H	68	161–162	4.32	4.43	E
N′-Methylpiperazino	H	65	254–256	8.63	8.51	E
Pyrrolidino	H	81	206–208	4.74	4.62	E
Pyrrolino	H	72	188–189	4.77	4.63	E
N,N-Diallylamino	H	80	124–126	4.36	4.30	E
2,6-Dimethylmorpholino	H	62	201–205	4.13	4.00	E
N,N-Dimethylamino	H	61	160–163	5.20	5.39	E
N,N-Diethylamino	H	70	179–181	4.71	4.53	E

[a] All melting points are corrected.

precipitate of alkylamine hydrochloride was filtered. The filtrate was partially evaporated and the amide was induced to crystallize by the addition of petroleum ether. The crystals were collected, dried, and recrystallized.

Procedure D

Ethyl N,N-Diphenyldiphenamate.—To a solution of 22 m*M* of ethyl diphenoyl chloride in 25 ml. of dry benzene was added 80 m*M* of dipnenylamine dissolved in 25 ml. of dry benzene. The mixture was refluxed for 10 hours; then the benzene solvent was removed under vacuum. The resulting residue was cooled overnight and then washed with cold methyl alcohol. The gummy solid was recrystallized from an aqueous methanol mixture to produce white crystals.

Procedure E

N,N-Dialkyldiphenamic Acid.—One-hundred millimoles of N,N-dialkylamine was added slowly with rapid stirring to 22 m*M* of diphenic anhydride. Heat was evolved and the mixture turned a brown color. Upon cooling, the reaction mixture was washed with ether to remove the excess amine. The residue was then dissolved in water and filtered. The filtrate was diluted with 150 ml. of water and then acidified with 10% hydrochloric acid. Upon standing, a precipitate was formed which could be further purified by recrystallizing from ethyl alcohol and water.

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Comparison of Extractive Procedures for Digitalis

By BERNARD R. LETASSY† and C. LEE HUYCK‡

The shearing action of the Premier colloid mill and the Eppenbach Homo-Mixer was applied to a mixture of digitalis powder and its extracting liquid in order to determine whether these pieces of equipment might be superior to the percolator for extraction of the active principles. The official method was used as the control procedure and simple agitation as an intermediate procedure. Data presented show order of effectiveness.

A MORE efficient extraction of the active principles of digitalis has been the target of numerous investigators. Bay and Gisvold (1) destroyed the enzymes in fresh digitalis leaves by heating the ground leaves to 70° before attempting extraction. In a later investigation Hopponen and Gisvold (2) found that quick freezing of the freshly harvested digitalis leaves retarded enzyme action and kept the glycoside content at a maximum until extracted. In an extension of this work (3) a maximum yield was obtained when the aqueous extraction represented two per cent of the drug on a dry leaf weight basis. Eighty-seven per cent of the activity could be extracted in this way. Aqueous extraction possesses several disadvantages, however. One disadvantage is hydrolysis of the active principle with a subsequent lower yield of glycosides. Another disadvantage is the glycosides are less soluble in water than they are in alcohol.

Kušević (4) made a comparative study of maceration, percolation, agitation, and digestion in an attempt to determine whether a more effective method for extraction of digitalis could be found. The digestion method with dilute alcohol at 60° gave the best results of the methods tested.

A somewhat different approach to the problem of extraction was taken by Dean, *et al.* (5), who demonstrated that it is possible to prepare tinctures in a few minutes, whereas the conventional methods require several days. This was accomplished by passing a suspension of the powdered drug in menstruum through a colloid mill. Using belladonna leaf, a 5-minute maceration yielded 37.2 per cent of the desired components. The percentage of yield increased to 99.5 per cent when the mixture of leaf and menstruum was circulated in a colloid mill continuously for 5 minutes.

EXPERIMENTAL

Using the milling method described by Dean, *et al.* (5), as a guide an attempt was made to increase the yield obtained by the official method for extraction of digitalis glycosides. In addition to the colloid mill, three other pieces of equipment were utilized: the Eppenbach Homo-Mixer, simple agitation in a screw-top jar; and the percolator (by the U.S.P. method which served as a control). Agitation in a jar provided an intermediate method to determine whether simple agitation had any effect on improving the extraction of the macerated leaf.

Procedure.—Four 100-gram portions of 80-mesh *Digitalis purpurea* powder were made evenly and distinctly damp with the menstruum (4 volumes alcohol and 1 volume water) and allowed to stand for 15 minutes. The control portion was packed in a cylindrical percolator. All four portions were allowed to macerate for 24 hours with additional menstruum.

At the end of the maceration period a suspension of the first portion was passed through the Premier colloid mill, type U-B7, using a setting of 0.005 inch between rotor and stator. The suspension was filtered and the marc was washed with menstruum until the filtrate measured 1,000 ml. A suspension of the second portion was subjected to the Eppenbach Homo-Mixer, type CS, for 5 minutes at maximum speed without loss of liquid and filtered through paper. The marc was washed with menstruum to volume. The third portion was shaken vigorously for 5 minutes in a one-gallon screw-top jar and filtered through paper. Likewise the marc was washed with menstruum to volume.

In order to insure accuracy, the entire process was repeated with another four 100-Gm. portions of the same batch of powder. The resulting tinctures were assayed by the chemical method of Bell and Krantz (6).

TABLE I.—PERCENTAGE STRENGTH OF TINCTURES PREPARED BY THE VARIOUS METHODS OF EXTRACTION IN TERMS OF U.S.P. REFERENCE STANDARD TINCTURE

Method	1st	2nd	Average
U.S.P. XV	88.5	89.7	89.1
Eppenbach Homo-Mixer	78.8	65.0	71.9
Premier colloid mill	73.5	69.0	71.2
Simple agitation	63.4	64.0	63.7

Table I reveals that percolation of the macerated digitalis leaf is superior to the other extraction methods. Probably the most important factor in favor of the percolation method is the fact that fresh menstruum is continually passing around the particles of the drug. Although this factor also took place in the modified methods where the marc was continually washed on the filter paper, it took place less efficiently. Table I also reveals the order in which the glycosides were extracted when percolation was not a factor. The Homo-Mixer gave approximately the same results as the colloid mill, and both were better than simple agitation.

SUMMARY

An attempt to increase the yield of digitalis glycosides by applying high speed shearing to a mixture of powdered drug and menstruum in place of percolation by the U.S.P. method was made. Shearing was obtained by two pieces of equipment

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commonly found in the manufacturing laboratory: the Premier colloid mill and the Eppenbach Homo-Mixer. Simple agitation in a screw-top jar was used as an intermediate procedure. Duplicate experiments (using these pieces of equipment) show the official method of extraction of digitalis (using the percolator) to be superior to methods using the other pieces of equipment tested.

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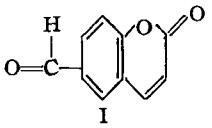
Antitubercular Activity of Some Aromatic Aldehyde and Ketone Derivatives

By AJIT K. DATTA† and T. C. DANIELS

The semicarbazones, para-nitrobenzoyl hydrazones and isonicotinyl hydrazones of the unsubstituted and three substituted 4-phenyl-2-oxo-3-butenoic acid and 6-formyl-1,2-benzpyrone were prepared and the toxicities and *in vitro* antitubercular activities measured. Three isonicotinyl hydrazones show the same order of antitubercular activity as isonicotinic acid hydrazide.

THE WELL KNOWN antitubercular activity of thiosemicarbazones, *p*-nitrobenzoyl hydrazones, and isonicotinyl hydrazones of a number of substituted and α,β-unsaturated aromatic aldehydes (1, 6) and ketones (2) made it of interest to prepare the corresponding derivatives of 6-formyl-1,2-benzpyrone (I) and of 4-phenyl-2-oxo-3-butenoic acid (II; R_1, R_2 = H), the latter possessing the additional advantage of affording water-soluble salts.



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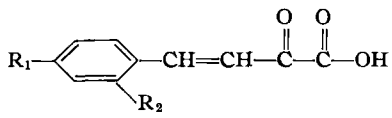

II

In view of the interesting antitubercular activity of the isonicotinyl hydrazone of II, the corresponding *o*-chloro, *o*-methoxy, and *p*-methoxy substituted derivatives of II were prepared for comparison and are described in Table I.

In vitro antitubercular activity was determined against *Mycobacterium tuberculosis* var. *hominus* strain H 37 RV by the serial dilution technique, using the modified Dubos medium (3). The results are shown in Table II.

Acute toxicity of the compounds was determined using intraperitoneal injections of suspensions in 6% acacia in Webster strain mice of both sexes (average weight 20 Gm.). The results are given in Table III.

EXPERIMENTAL[1]

6-Formyl-1,2-benzpyrone.—Prepared by the method of Sen and Chakravarti (4), the compound melted at 189° (lit. m.p. 187–189°) (4).

6-Formyl-1,2-benzpyrone Thiosemicarbazone.—A mixture of 0.8 Gm. of 6-formyl-1,2-benzpyrone in 35 ml. of 80% ethanol, 0.45 Gm. of thiosemicarbazide, and 1 Gm. of sodium acetate was refluxed on a steam cone for 1 hour. The reaction product was recrystallized from dilute alcohol. One gram (85% yield) of a cream-colored crystalline product was obtained. The compound sublimes without melting at 300°.

Anal.—Calcd. for $C_{11}H_9N_3O_2S$: N, 17.0. Found: N, 16.79.

6-Formyl-1,2-benzpyrone *p*-Nitrobenzoyl Hydrazone.—A mixture of 0.9 Gm. of 6-formyl-1,2-benzpyrone in 35 ml. of 80% ethanol and 0.9 Gm. of *p*-nitrobenzoyl hydrazide in 20 ml. of ethanol was refluxed for 1.5 hours on a steam bath. The reaction product was recrystallized from 30% alcohol from which 1.5 Gm. (90% yield) of lemon-yellow crystals was obtained, m.p. 314–315°.

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Anal.—Calcd. for $C_{16}H_{10}N_3O$: N, 14.33. Found: N, 14.53.

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The authors are indebted to Professor John C. Craig for his helpful advice and suggestions.
This paper is a portion of a thesis submitted by Ajit K. Datta to the Graduate Division, University of California, in partial fulfillment of Doctor of Philosophy degree requirements.

† Present address: Hoechst Pharmaceutical Co., Scientific Department, Calcutta 13, India.

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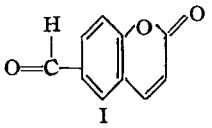

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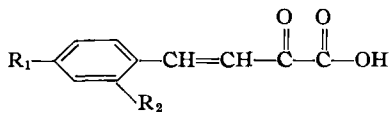

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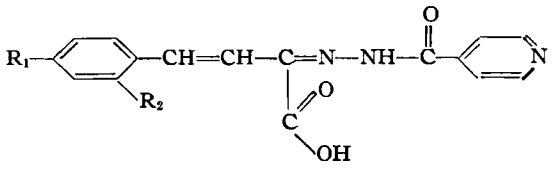
TABLE I.—UNSUBSTITUTED AND SUBSTITUTED 4-PHENYL-2-OXO-3-BUTENOIC ACID ISONICOTINYL HYDRAZONES



R_1	R_2	Yield, %	M.p., °C.	Formula	Found, N %	Calcd., N %
H	H	90	308	$C_{16}H_{13}N_3O_3$	14.50	14.23
H	Me—O	90	185.5	$C_{17}H_{15}N_3O_4$	12.89	12.96
Me—O	H	92	201–202	$C_{17}H_{15}N_3O_4$	12.79	12.96
H	Cl	85	190 to 190.5	$C_{16}H_{12}N_3O_3Cl$	12.81	12.74

TABLE II.—ANTITUBERCULAR ACTIVITY

Compound	Required for Inhibition of Growth, min. mol. concn.
4-Phenyl-2-oxo-3-butenoic acid	
-thiosemicarbazone	10^{-4}
-*p*-nitrobenzoyl hydrazone	10^{-3}
-isonicotinyl hydrazone	10^{-5}
4-(*p*-Methoxyphenyl)-2-oxo-3-butenoic acid	
-thiosemicarbazone	10^{-4}
-isonicotinyl hydrazone	10^{-5}
-*p*-nitrobenzoyl hydrazone	10^{-3}
4-(*o*-Methoxyphenyl)-2-oxo-3-butenoic acid	
-isonicotinyl hydrazone	10^{-5}
4-*o*-Chlorophenyl-2-oxo-3-butenoic acid	
-isonicotinyl hydrazone	10^{-5}
6-Formyl-1,2-benzpyrone	
-semicarbazone	10^{-4}
-*p*-nitrobenzoyl hydrazone	10^{-3}
-isonicotinyl hydrazone	10^{-5}
Isonicotinic acid hydrazide	10^{-5}

4-Phenyl-2-oxo-1-3-butenoic Acid.—Prepared by the method of Erlenmeyer (5), the compound melted at 50° (lit. m.p. 53°) (5).

Anal.—Calcd. for $C_{10}H_8O_3$: C, 68.18; H, 4.54. Found: C, 68.33; H, 4.72.

4-*o*-Methoxyphenyl-2-oxo-3-butenoic Acid.—Prepared by the same procedure, this acid was obtained as light-orange needles in 50% yield, m.p. 133–134°.

Anal.—Calcd. for $C_{17}H_{15}N_3O_4$: N, 12.96. Found: 12.89.

4-*p*-Methoxyphenyl-2-oxo-3-butenoic Acid.—By the method described, this formed yellow needles in 62% yield, m.p. 126° dec. (lit. m.p. 131°) (7).

4-*o*-Chlorophenyl-2-oxo-3-butenoic Acid.—Prepared as described above, this acid was obtained in 62% yield as light-yellow crystals, m.p. 195° dec.

Anal.—Calcd. for $C_{16}H_{12}N_3O_3Cl$: N, 12.74. Found: N, 12.81.

TABLE III.—ACUTE TOXICITY IN MICE (INTRAPERITONEALLY)

Compound	LD_{50}, mg./Kg.
6-Formyl-1,2-benzpyrone	
-thiosemicarbazone	350
-*p*-nitrobenzoyl hydrazone	>350
-isonicotinyl hydrazone	>500
4-Phenyl-2-oxo-3-butenoic acid	
-isonicotinyl hydrazone[a]	>750

[a] The LD_{50} of all compounds listed in Table I was greater than 750.

4-Phenyl-2-oxo-3-butenoic Acid Thiosemicarbazone.—Prepared as described above, this was obtained in 75% yield as yellow needles, m.p. 191° dec.

Anal.—Calcd. for $C_{11}H_{11}O_2N_3S$: N, 16.87. Found: N, 17.02.

4-Phenyl-2-oxo-3-butenoic Acid *p*-Nitrobenzoyl Hydrazone.—By the method described, this formed greenish-yellow needles in 70% yield, m.p. 231°.

Anal.—Calcd. for $C_{17}H_{13}O_5N_3$: N, 12.36. Found: N, 12.49.

4-Phenyl-2-oxo-3-butenoic Acid Isonicotinyl Hydrazone.—Prepared as described under *6-Formyl-1,2-benzpyrone Isonicotinyl Hydrazone.* The compound recrystallized from 33% alcohol was obtained in 90% yield as light cream-colored needles, m.p. 308°.

Anal.—Calcd. for $C_{16}H_{13}N_3O_3$: N, 14.23. Found: N, 14.50.

Three Phenyl Substituted Isonicotinyl Hydrazones of 4-Phenyl-2-oxo-3-butenoic Acid.—These were prepared following the same procedure and are listed under Table I.

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Light—An Essential Factor in the Trihydroxyindole-Spectrophotofluorometric Assay of Norepinephrine

By LINCOLN CHIN, ALBERT L. PICCHIONI, and RICHARD F. CHILDS

When iodine is used as the oxidant in the spectrophotofluorometric assay of norepinephrine, conversion of the oxidation product (noradrenochrome) to the fluorophore (noradrenolutine) requires the presence of adequate light. At a light intensity of 105 foot-candles, a period of 30 minutes is required for maximal development of fluorescence. With the use of intense light (sun light: 8400 foot-candles), maximal development of fluorescence was obtained in 2 minutes. At a light intensity of 40 foot-candles or less, the development of fluorescence was incomplete even when the conversion reaction was allowed the prescribed time of 45 minutes. It is apparent that light is an important factor in the conversion of noradrenochrome to noradrenolutine. Consistent and reproducible results are obtained only when this part of the assay is carried out under an adequate intensity of light for a suitable length of time.

THE SPECTROPHOTOFLUOROMETRIC method for the assay of norepinephrine described by Shore and Olin (1) involves initial oxidation of the amine to noradrenochrome by iodine at a weakly acidic pH. Noradrenochrome is then rearranged to a highly fluorescent trihydroxyindole (THI) derivative, noradrenolutine, by an alkaline ascorbate solution. Forty-five minutes are allowed for maximal development of fluorescence; the solution is then placed in a spectrophotofluorometer and activated at 400 mμ and the resulting fluorescence read at 520 mμ.

During the course of some early norepinephrine assays in this laboratory, it was observed that fluctuation of daylight in the room, assay at different times of day, and shielding of test tubes from light during the THI reaction yielded erratic results. In the case of samples that were exposed to strong light, development of fluorescence was complete, as suggested by a rapid excursion of the galvanometer indicator-needle to the peak reading. However, in the case of samples that were exposed to weak light or were shielded from bright light, development of fluorescence was incomplete, as suggested by an initial rapid sweep of the indicator to an intermediate point on the galvanometer scale which was then followed by a slower increase in the reading. When a continuous reading was made of these latter solutions, the indicator drifted slowly and reached a peak reading only after approximately 30 minutes, during which time the sample in the spectrophotofluorometer was continuously irradiated by the activating lamp. These early observations suggest that light has a definite effect on this assay. Because there is a paucity of information in the literature regarding the influence of light on this analysis for norepinephrine, studies were undertaken to investigate further the role of light in the procedure.

EXPERIMENTAL

Norepinephrine,[1] 0.1 mcg./ml. in 0.01 *N* HCl, was used throughout this investigation. The chemical reactions were carried out in the manner described by Shore and Olin (1). In one study, following addition of alkaline ascorbate, test tubes of the sample were exposed to varying intensities of light for a standard period of 45 minutes. In another study, test tubes of the sample were exposed to a standard light intensity for various periods of time. In addition, other test tubes of sample were exposed to direct sunlight (*ca.* 8400 foot-candles) to determine the effect of an intense light on the THI reaction. Measurements of fluorescence were made with the Aminco-Bowman spectrophotofluorometer.[2]

RESULTS

As illustrated in Fig. 1, completion of noradrenochrome rearrangement requires an adequate light intensity. When the reaction was allowed to take place in the dark for 45 minutes, a negligible amount of fluorescence developed. However, when the reaction was permitted to take place in graded intensities of light, relatively greater degrees of fluorescence developed up to a maximal level. Light intensity of 105 foot-candles was sufficient for producing maximal fluorescence in 45 minutes of exposure; a greater intensity yielded no additional increase in fluorescence. Figure 2 shows that with a light intensity of 105 foot-candles, adequate time is required for completion of the THI reaction. Within the initial 15 minutes, following addition of alkaline ascorbate, there was a rapid development of fluorescence. By 20 minutes the rate of reaction had diminished appreciably and by 30 minutes fluorescence had essentially reached its peak. Readings made after longer intervals of exposure were not significantly different from those obtained at the 30-minute period. In the study which utilized an intense light source, samples that were exposed to direct sunlight showed maximal fluorescence after 2 minutes of exposure. When the samples that had been kept in the dark or exposed to dim light were subsequently exposed to sunlight for 2 minutes, they developed a degree of fluorescence comparable to that seen in the samples exposed for 45 minutes to artificial light of 105 foot-candles. On the other hand, samples that had been allowed

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[1] Norepinephrine bitartrate, supplied by Winthrop Laboratories, was used to prepare the norepinephrine solution. The concentration is calculated and expressed as norepinephrine base.

[2] The galvanometer readings of samples, in which development of fluorescence was incomplete, were made at the end of the initial rapid sweep of the indicator before the onset of its slow drift to a peak value.

to react for 45 minutes in artificial light of 105 foot-candles developed no additional fluorescence upon further exposure to direct sunlight for 2 minutes.

DISCUSSION

Lund (2), who employed manganese dioxide as the oxidizing agent in the formation of noradrenochrome, indicates that rearrangement of this aminochrome to noradrenolutine occurs almost instantaneously following addition of alkali. Maynert and Klingman (3) report that if potassium ferricyanide is substituted for iodine in the assay of catecholamines, fluorescence may be measured 5–10 minutes after addition of alkali. Udenfriend (4) also indicates that when potassium ferricyanide is used as the oxidizing agent, the conversion of aminochromes to fluorophores is completed very rapidly, 1–2 minutes after addition of alkali. On the other hand, as indicated by the above investigators (3, 4) iodine-oxidized norepinephrine requires a longer period for conversion from noradrenochrome to noradrenolutine. In view of the apparent difference in the rate of rearrangement associated with the use of various oxidizing agents and of the observations made in this laboratory, it is postulated that rapid conversion of noradrenochrome to the fluorophore requires either a metal catalyst or radiant energy. When manganese dioxide or potassium ferricyanide is used as the oxidizing agent, it is conceivable that metallic ions may act as catalysts during the THI reaction. Whereas, when iodine is used as the oxidizing agent, light may supply the energy for accelerating the formation of noradrenolutine; if the intensity of light is exceptionally great (*e.g.*, sunlight), rearrangement may occur at a rate comparable to that of the assay in which one of the metallic salts is used.

It is of interest that reports on the effect of light on the ethylenediamine procedure for the estimation of norepinephrine (5, 6) indicate that daylight causes a *loss* of fluorescence. On the basis of this finding it would appear logical to shield the test tubes from light during the THI reaction in the assay procedure of Shore and Olin (1). However, the present observations demonstrate that when iodine is employed as the oxidant an adequate light intensity is imperative during the THI reaction. Insufficient light during this phase would result in erratic readings; this may account for some of the dissatisfaction voiced by some investigators for the THI-spectrophotofluorometric assay of norepinephrine. On the other hand, one group of investigators (7), who employed iodine as the oxidizing agent, reports 20 minutes (rather than 45 minutes) as adequate for completion of the THI reaction. The shorter time period used by these workers could be explained on the basis of a relatively bright illumination in the laboratory.

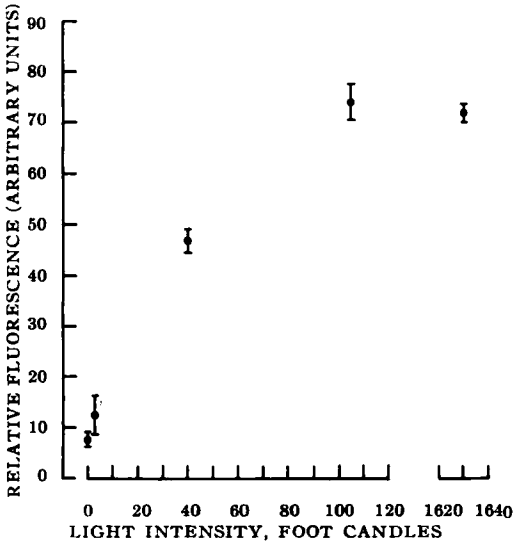

Fig. 1.—Relative fluorescence of norepinephrine samples following oxidation, addition of alkaline ascorbate, and exposure to graded intensities of light for 45 min. Four or more samples were used for each determination. Vertical bracketed lines indicate 95% confidence limits. The reaction at 1630 foot-candles was carried out under sunlight partially shaded with layers of cotton gauze to produce this light intensity; other reactions were carried out in darkness or artificial light (fluorescent light).

CONCLUSION

The data reported herein show that when iodine is employed as the oxidizing agent in the spectrophotofluorometric assay of norepinephrine, light is essential for the conversion of noradrenochrome to noradrenolutine. This finding supports the warning recently advanced by Udenfriend (4) (with respect to the THI reaction) that when iodine is used as the oxidizing agent shielding the samples from light will produce erratic results. It is obvious that consistent and reproducible results can not be obtained unless the THI reaction is allowed to take place under an adequate intensity of light for a suitable period of time.

ADDENDUM

Subsequent to completion of the present studies, Sloan and co-workers (8) reported that following the

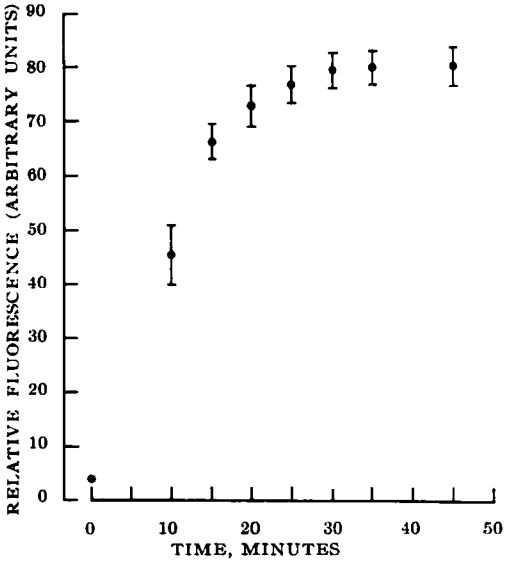

Fig. 2. –Relative fluorescence of norepinephrine samples following oxidation, addition of alkaline ascorbate, and exposure to a standard light intensity of 105 foot-candles (fluorescent light) for various time intervals. Four samples were used for each determination except that one sample was employed for the zero-time determination. Vertical bracketed lines indicate 95% confidence limits.

addition of alkaline ascorbate solution the reaction is extremely photosensitive. These investigators avoided erratic results by placing the tubes of samples inside a box which contains two General Electric 20-watt ultraviolet black light bulbs. They found that rigid control of the lighting conditions enhanced the intensity of fluorescence and improved reproducibility. This finding further supports the contention that light is an essential component in the spectrophotofluorometric assay of norepinephrine during the THI reaction.

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Cyclized Substituted Thioureas III

1-Substituted-tetrazolines-5-thiones

By RONALD E. ORTH

A convenient synthesis and purification of some 1-substituted-tetrazoline-5-thiones is accomplished. Decreased per cent yields with increased chain length lends some credence to the probability that steric hindrance occurs in the ring closing step. The infrared studies show identifying bands for —N=C=S, C=S, cyclic —N—N=N—, and the tetrazole ring. Low toxicity for these compounds is indicated.

QUALIFIED success with propylthiouracil (1) and methimazole (2) during hyperthyroid therapy has led to the synthesis of chemically related compounds and to their subsequent *in vitro* and *in vivo* trials. Countless antibacterial and tuberculostatic screening programs have also been carried out using compounds which embody the thiourea moiety.

Some 1-alkyltetrazoline-5-thiones were prepared by synthesizing the methyl ester of *N*-alkyldithiocarbamic acid and refluxing it with sodium azide (3). A more convenient method consists of preparing the sodium *N*-alkyl dithiocarbamate from carbon disulfide, the corresponding amine, and sodium hydroxide. Ethyl chloroformate and the dithiocarbamate give ethyl *N*-alkyldithiocarbamate, which upon decomposition yields carbonyl sulfide, ethanol, and the desired alkyl isothiocyanate (R—N=C=S). The 1-substituted-tetrazoline-5-thiones were isolated following reflux of the isothiocyanate with sodium azide and acidification. The lower yields with increased chain length suggests that there is a steric effect since it has been demonstrated that 1-aryl substituents increases the yields appreciably (4).

Tetrazole and its substitution products usually show only a small amount of end absorption in the ultraviolet region of the spectrum (240–250 mμ) (5). Otting found that tetrazole gave large peaks at 1520, 1270, 1160, 1100, 1020, 915, and 670 cm.$^{-1}$ and a number of characteristic small peaks (6). Lieber, *et al.*, found some 5-substituted tetrazoles to give results interpreted as tetrazole ring absorption bands in the 8.9 to 10 μ region (7). In later work with 1-aryl-tetrazoline-5-thiones more definite tetrazole skeletal assignments were demonstrated. The bands were found at approximately 1100, 1080, 1045, 1020, and 990 cm.$^{-1}$ Frequencies were also assigned for N—C=S, C=S, and cyclic —N—N=N— linkages (8). The spectra for the 1-substituted-tetrazoline-5-thiones summarized in Table I closely approximate the Lieber assigned values and support his hypothesis showing the presence of sulfur in the thione form. Characteristic sulfhydryl bands were not seen for any of the compounds tested from this series. The results of this investigation emphasize the uniformity of the data obtained using 1-aryl and 1-alkyl substituents in the tetrazoline-5-thione system.

LD_{50}'s of 207 ± 27 mg./Kg. and 215 ± 23 mg./Kg. body weight were obtained for 1-methyl- and 1-isopropyltetrazoline-5-thiones, respectively, using five groups per compound, each group having 20 young male white mice.

EXPERIMENTAL

1-Substituted-Tetrazoline-5-thiones.—Three-tenths mole (19.5 Gm.) of sodium azide is dissolved in 300 ml. of water and filtered into a 500 ml. round-bottom flask and two-tenths mole of an isothiocyanate is added, followed by refluxing. Cease the operation and remove any unreacted isothiocyanate by ether extraction when the green reaction mixture turns colorless. Acidify the aqueous solution to a pH of 3 with HCl, filter, and extract the filtrate with ether. Wash the ether solution with small portions of ice water, dry over anhydrous sodium sulfate, and decolorize with activated charcoal. The dried acidic ether extract is evaporated to a yellow-brown oil, which in turn forms pale yellow crystals, using the Rinco rotary evaporator and steam bath. These crystals are blotted dry on filter paper, and recrystallized from 100–115° petroleum ether.

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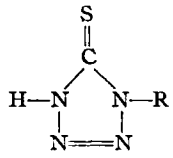

R	M.p. °C	Yield,[a] %	Nitrogen, % Calcd.	Nitrogen, % Obs.[b]	Infrared Spectra[c, e, f] —N—C=S	C=S	Cyclic —N—N=N	Tetrazole Skeletal Bands			
Methyl	125–26	52	48.2	48.5	1510(m)	1350(m)	1300(m)		1066(w)	1042(s)	
Ethyl	50	37	...	...	1500(w)		1275(s)	1088(s)		1045(s)	983(m)
n-Propyl	77–78	24	38.8	40.0	1510(s)	1350(s)	1290(m)	1112(m)		1050(s)	995(s)
iso-Propyl[d]	89–90	34	38.8	38.7							
Allyl	153	22	39.5	39.5	1500(s)	1350(m)	1304(m)		1075(m)	1053(s)	990(s)
iso-Butyl[d]	64–65	25	35.4	35.4							
t-Butyl[d]	98–99	29	35.4	35.4							
Benzyl	148	87	31.1	31.9	1495(m)		1290(w)	1090(w)		1053(m)	

a Average yield based on two runs per compound. *b* Weiler and Strauss, Microanalytical laboratory, Oxford, England (average of two runs). *c* Dr. Paul R. Caudill, College of Agriculture, University of Kentucky, Lexington. *d* Not subjected to infrared studies. *e* Perkin Elmer 21 instrument used; Nujol phase; filter out; NaCl prism; 927 program; speed 1 μ/min.: response 1; suppression 0. *f* Key: (s) = strong band; (m) = medium band; (w) = weak band.

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Determination of Ergotamine and Ergotaminine in Pharmaceutical Preparations

By THOMAS G. ALEXANDER

Ergotamine and its diastereoisomer, ergotaminine, are separated from each other and from other ingredients by column chromatography. The isolated fractions are then assayed by the classical van Urk method. Identity and purity of the fractions are confirmed by paper chromatography. A number of commercial products were analyzed successfully.

ERGOTAMINE in solution forms an equilibrium mixture of the diastereoisomers, ergotamine, and ergotaminine. This study was undertaken to develop a practical quantitative method for the assay of both ergotamine and ergotaminine in injection solutions. Of the different techniques that have been described for this purpose, column chromatography appeared to be the best suited. Larger amounts of alkaloid can be recovered more efficiently on a column than with methods involving the extraction of spots from paper or thin-layer chromatograms (1–3). Yet large samples are not required as with the polarimetric methods (4). Also a column chromatographic technique does not require the use of as much time and specialized glassware as would one involving countercurrent extraction (5).

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The author thanks the following persons for their cooperative assistance: J. Kottemann, Division of Pharmaceutical Chemistry, and A. Major, Jr., Bureau of Field Administration, both of the Food and Drug Administration.

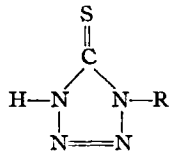
The method presented involves partition chromatography using a column prepared by adsorbing 1:4 citric acid solution on siliceous earth. This column extracts ergotamine from a chloroform solution containing both ergotamine and ergotaminine. The latter passes through in the effluent and is assayed. Ergotamine is recovered by chloroform extraction of the extruded column. This procedure was submitted to the Committee on Revision of the "United States Pharmacopeia" and it has been incorporated into the Monograph for Ergotamine Tartrate Injection. The procedural details are contained in this revised monograph (6).

To analyze ergotamine salts and simple tablet mixtures, weighed portions are dissolved in or triturated with 1:100 tartaric acid solution; the sample is then analyzed in the manner described in the revised monograph for injections.

EXPERIMENTAL AND DISCUSSION

In following the methods described by Berg (7) and van de Langerijt (8) involving the use of benzene as eluent, both ergotamine and ergotaminine rapidly epimerized and deteriorated. Carless (9)

TABLE I.—1-SUBSTITUTED-TETRAZOLINE-5-THIONES



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The method presented involves partition chromatography using a column prepared by adsorbing 1:4 citric acid solution on siliceous earth. This column extracts ergotamine from a chloroform solution containing both ergotamine and ergotaminine. The latter passes through in the effluent and is assayed. Ergotamine is recovered by chloroform extraction of the extruded column. This procedure was submitted to the Committee on Revision of the "United States Pharmacopeia" and it has been incorporated into the Monograph for Ergotamine Tartrate Injection. The procedural details are contained in this revised monograph (6).

To analyze ergotamine salts and simple tablet mixtures, weighed portions are dissolved in or triturated with 1:100 tartaric acid solution; the sample is then analyzed in the manner described in the revised monograph for injections.

EXPERIMENTAL AND DISCUSSION

In following the methods described by Berg (7) and van de Langerijt (8) involving the use of benzene as eluent, both ergotamine and ergotaminine rapidly epimerized and deteriorated. Carless (9)

TABLE I.—ASSAYS OF SIMULATED INJECTION SOLUTIONS

Sample	Isomer[a]	mg. Added (as tartrates)	mg. Found	Levo Added, %	Levo Calcd., %	Recovery of Both Isomers, %
A	Levo	5.11	5.14	100	99.0	101.6
	Dextro	0	0.05	...	...	...
B	Levo	4.09	3.94	81.3	81.6	96.0
	Dextro	0.94	0.89	...	...	...
C	Levo	2.66	2.56	54.1	52.6	99.0
	Dextro	2.26	2.31	...	...	...
D	Levo	0	0.14	0	3.0	100.6
	Dextro	4.69	4.58	...	...	...

[a] The levorotatory alkaloid is ergotamine. The dextrorotatory alkaloid is ergotaminine.

TABLE II.—ASSAY RESULTS

Sample	Ingredients Other Than Ergotamine	Per Cent of Declared, or of Amount Added — Analyst I *l*	Analyst I *d*	Analyst II *l*	Analyst II *d*	Analyst III *l*	Analyst III *d*
Simulated Injection	None	67.5	33.4	68.0	32.3	...	...
		66.9	33.3	68.9	32.1	...	...
Commercial Tablets	Phenobarbital Belladona salts	74.0[a]	10.6[a]	...	...	75.2[a]	13.0[a]
		71.8	15.3	...	...	...	...
Commercial Tablets	Phenobarbital Caffeine	69.3[a]	19.0[a]	...	...	71.1[a]	19.8[a]
		69.5	20.5	...	...	...	...
Ergotamine Tartrate	...	75.9	14.2[b]	...	...	...	...
		77.0	14.1[b]	...	...	...	...
Commercial Suppositories	Caffeine	61.5	26.5	(each line of different batch)			
	Phenobarbital sodium	65.0	22.0	...	...	...	...
Cocoa-butter	Belladona salt	18.0	52.0	...	...	...	...
Simulated Suppositories Wecobee NC 6112[c]	Caffeine Acetophenetidin Belladona salt	94.8	2.4	...	...	...	...
				...	...	...	...

[a] These results were obtained on individual whole tablets. [b] This ergotamine tartrate was unusually impure. The fact that only about 90% of the sample used was received would indicate that either part of the material was retained by the first column or if it came through in either the ergotamine or ergotaminine fractions, it did not respond to the color reaction. In either case, it could not be closely related to ergotamine. Infrared data indicated that the material in the *dextro* fraction was not ergotamine but might have been of the ergotoxine group or one of the "aci-ergot" alkaloids (13). [c] The author is grateful to E. F. Drew and Co. for supplying this hydrogenated vegetable oil suppository base.

recovered only 80% of the ergotamine in pyridine-ether effluents from columns of cellulose treated with McIlvaine citrate-phosphate buffer. In attempting to improve upon Carless' method, it was learned that both phosphate salts and pyridine have detrimental effects on ergotamine.

The sharpness with which citric acid columns will resolve mixtures of ergotamine and ergotaminine in chloroform varies with the strength of the acid. Columns prepared with strong 1:3 citric acid solution will sharply separate the diastereoisomers, but an excessive volume of effluent is required to elute completely the ergotaminine. With columns using weaker acid solution, 1:5, the ergotamine and ergotaminine fractions overlap. The column developed (9 Gm. siliceous earth plus 7 ml. of 1:4 citric acid solution) retains ergotamine, while about 97.5% of the ergotaminine appeared in the first 100 ml. and another 1 or 1.5% was obtained in the next 100 ml. Recoveries of 97 or 98% of the ergotamine were obtained by extraction of the extruded and alkalized column. The completeness of these separations was also checked by paper chromatography, using a modified version (10) of Stoll and Rüegger's reversed-phased method (11). After aliquots of the two chloroform extracts were removed for assay, the remaining portions were evaporated to a concentration suitable for spotting on paper chromatograms. Ergotamine was not detected in the ergotaminine fractions tested. Occasionally, trace amounts of ergotaminine were detected in the ergotamine fraction.

To check further the validity of the method, four simulated injection solutions were prepared, using ergotamine bitartrate freshly recrystallized from methanol and ergotaminine prepared by Stoll's method (12). These solutions were then assayed promptly. The results are presented in Table I.

ADAPTATION TO COMPLEX MIXTURES

For the analysis of ergotamine tartrate tablets and suppositories containing caffeine, acetophenetidin, barbiturates, and belladona alkaloids[1] it is necessary that the ergot alkaloids first be separated from the other ingredients prior to the isolation of ergotamine from ergotaminine. For this purpose, a previously published method was found to be suitable (10), whereby ergotamine and ergotaminine are obtained together in chloroform solution isolated from the other nitrogenous bases. This chloroform solution is then passed onto the citric acid column and the analysis continued as with the simple formulations.

RESULTS

Several mixtures were prepared to simulate commercial products. These and several commercial preparations were analyzed. The assay results are

[1] This method is not applicable to preparations containing cyclizine.

presented in Table II. Identity and purity checks by paper chromatography showed that (except as noted) no ergot alkaloids other than ergotamine or ergotaminine were detected in the products. In some older samples with low assay values, brown spots appeared at $R_f = 0$ on the paper chromatogram of the ergotaminine fraction. These probably represent ergotamine that has oxidized or otherwise deteriorated.

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Determination of Zinc in Insulin Preparations

By BENNIE ZAK and JERRY S. COHEN

A description of an accurate absorptiometric technique for the determination of zinc in insulin is presented. It involves differential demasking of zinc in the presence of contaminating interferences such as copper and iron, followed by a reaction with the color reagent, zincon. It appears to be a simple yet useful process for quality control.

AN IMPORTANT analytical phase for the pharmaceutical industry involves quality control of products (1). The determination of zinc in insulin preparations for quality control purposes is an assay which presents some difficulty. Included among the procedural techniques for this element are processes employing spectrophotometry (2, 3), polarography (4, 5), and chelatometric titrations (6, 7). The first two types of procedure involve selective dithizone extractions followed by colorimetric measurement and retrograde extraction followed by polarographic measurement. The titrimetric procedure with a complexone titrant becomes more difficult and subjective as the concentration of zinc decreases. One milliliter of 0.001 *N* ethylenediamine tetraacetic acid corresponds to 65.4 mcg. of zinc (8); some insulin preparations, such as the unmodified variety, contain only 6 mcg. of zinc per ml. A visual titration involving a two-color indicator, such as Eriochrome black T, is subject to serious shortcomings when the titrant becomes very dilute, and the indicator color-change represents a slow transition from one form to the other (9).

The procedure for insulin zinc analyses to be discussed here involves an absorptiometric technique without extraction. It is capable of achieving an accurate determination for zinc yielding high absorbance data on samples which are never greater than 1 ml., even for those preparations containing the smallest amounts of zinc (10, 11). In the case of zinc-insulin solutions, the required amount of material is a micro quantity.

EXPERIMENTAL

Reagents.—Borate buffer, pH 9.0: dissolve 31 Gm. of boric acid in metal-free distilled water, add 53 ml. of 4 *N* NaOH, and dilute the solution to a liter with any necessary adjustment of pH. Zincon color reagent: dissolve 130 mg. of zincon in 2 ml. of the detergent, acationox, and dilute the solution to 100 ml. with metal-free distilled water. Stock standard zinc solution (100 mg./L.): dissolve 100 mg. of zinc in a minimum amount of HCl and dilute the solution to a liter with metal-free distilled water. This stock is diluted with water so that 0–18 mcg. per aliquot was used as a working standard.

Procedure.—An aliquot containing approximately 6–18 mcg. of zinc was pipeted into a centrifuge tube and diluted to 3.0 ml. with metal-free distilled water. This aliquot ranged from 0.05 ml. for an 80-unit globin-zinc-insulin preparation to 1.0 ml. for a 40-unit unmodified insulin preparation. To the diluted insulin solution, 1.5 ml. of 1 *N* HCl was added to liberate the zinc from its protein binding site. Ten per cent trichloracetic acid (1.5 ml.) was then mixed in to precipitate the proteins. Trichloracetic acid may be capable of performing both functions, but the stronger acid ensures that the zinc will become unbound. After a 5-minute stand, the mixture was centrifuged, and a 4.0-ml. aliquot was transferred to a Coleman 19 mm. O.D.

TABLE I.—PRECISION OF RECOVERY OF ABSOLUTE QUANTITIES OF ZINC

3.0 Mcg.	5.0 Mcg.	7.0 Mcg.	9.0 Mcg.	12.0 Mcg.
3.0	4.9	6.8	8.9	12.3
3.0	5.0	6.8	8.8	11.9
2.9	4.8	6.9	9.0	11.9
2.9	5.0	6.9	9.0	12.3
3.1	5.0	7.1	9.0	12.3
3.1	4.9	6.8	8.9	12.3
3.0	5.0	6.9	9.2	11.9
3.1	4.9	7.1	8.8	12.0
3.0	5.0	7.0	9.0	11.9
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2.9	5.0	6.9	9.0	12.3
3.1	5.0	7.1	9.0	12.3
3.1	4.9	6.8	8.9	12.3
3.0	5.0	6.9	9.2	11.9
3.1	4.9	7.1	8.8	12.0
3.0	5.0	7.0	9.0	11.9
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cell. A quantity of 4.0 *N* NaOH was added to neutralize the acidity so the capacity of the buffer would not be exceeded (0.4 ml. was used in the described procedure). One milliliter of pH 9 buffer was added followed by 0.1 ml. of 3% NaCN solution to form complexes with the zinc and any contaminating copper and iron present in the sample (12) or in the reagents used. Zincon color reagent (0.6 ml.) was then added. Because the cyano complexes are too tightly bound to release the metals for reaction with zincon, each tube can be used as its own blank. The instrument was zeroed at 630 mμ with the tube, then 0.2 ml. of 60% chloral hydrate solution was quickly mixed into each tube of standard or sample to demask the zinc differentially. Within 1 minute after the final addition, the absorbance of each tube was obtained again at 630 mμ. Since the zinc cyanide complex breaks down first, contaminating trace metals do not interfere in the process.

DISCUSSION AND RESULTS

The self-blanking described under procedure was quite convenient and yielded accurate results. Slight variations in different blanks were not significant because the absorbance difference before and after demasking could be obtained precisely. Background color characteristics of samples did not appear to affect the answers and this was decidedly advantageous. Copper and iron are ubiquitous trace contaminants of reagents. It was felt therefore that masking them in a system wherein demasking occurred much later than for the desired zinc would ensure accurate determinations.

A precision and accuracy study was carried out using replication on several absolute concentrations of zinc. The findings are shown in Table I. The values obtained were quantitatively in accord with those values known to be present in every instance. Conclusions concerning the precision as well as the accuracy of the determinations were therefore obvious in this experiment.

Twenty lots of different types of insulin unknowns were analyzed for their zinc content by the described procedure. The preparations varied from approximately 6 mcg. of zinc per ml. to 240 mcg. per ml. The results of this blind spectrophotometric study were compared to an ethylenediamine tetraacetate titration technique carried out in another laboratory where neither laboratory knew the results obtained by the other in advance. The comparative values are shown in Table II. The values obtained for the spectrophotometric procedure compare well enough to the EDTA titration since visual titrations of low concentration constituents with two-color indicators such as the Eriochrome black T used here are difficult (13) even if screening is employed as a visual aid (14).

Different quantities of zinc were added to aliquots of each of several lots of insulin. Zinc determinations were carried out on the insulins where a prior analysis of the materials established the base values. Because the zinc contents for different types of insulin are of such divergent composition (6–240 mcg./ml.), it was necessary to vary the size of the starting sample to conform to the best spectrophotometric characteristics of the system. The values found for base plus addition were quantitative and these results make up Table III.

TABLE II.—BLIND COMPARISON OF TWO PROCEDURES FOR ZINC BY TWO LABORATORIES

Lot	Lab A[a]	Lab B[b]	Lot	Lab A[a]	Lab B[b]
1	6	6	11	10	9
2	13	12	12	11	9
3	6	6	13	22	18
4	7	6	14	23	18
5	8	6	15	21	18
6	14	12	16	126	120
7	15	12	17	247	240
8	11	9	18	122	120
9	22	18	19	247	240
10	11	9	20	247	240

[a] Described procedure. [b] Chelatometric titration, courtesy of E. R. Squibb and Sons, Technical Services Department.

TABLE III.—RECOVERY OF VARIOUS ADDITIONS OF ZINC TO INSULIN

Type of Insulin	Sample Size, ml.	Present, mcg./ml.	Added	Total Present	Total Found
Unmodified	0.50	13.5	3.0	9.8	9.4
Unmodified	0.50	13.5	3.0	9.8	9.4
Unmodified	0.50	13.5	6.0	12.8	12.8
Unmodified	0.50	13.5	6.0	12.8	12.5
Unmodified	0.50	13.5	9.0	15.8	16.0
Unmodified	0.50	13.5	9.0	15.8	15.4
NPH	0.10	23.3	3.0	5.3	5.1
NPH	0.10	23.3	6.0	8.3	8.1
NPH	0.10	23.3	9.0	11.3	10.8
NPH	0.25	23.3	3.0	8.8	8.4
NPH	0.25	23.3	6.0	11.8	11.6
NPH	0.25	23.3	9.0	14.8	13.8
Globin	0.05	122	3.0	9.1	9.4
Globuin	0.05	122	3.0	9.1	9.7
Globuin	0.05	122	6.0	12.1	12.4
Globin	0.05	122	6.0	12.1	12.2
Globin	0.05	122	9.0	15.1	15.4
Globin	0.05	122	9.0	15.1	15.4

SUMMARY

A procedure has been described for the spectrophotometric determination of the zinc content of various lots of insulin. This technique for quality control involves differential demasking as a means of precluding the effect of contaminating trace metals such as copper and iron. The process is simple, accurate, and capable of rapid analysis for large numbers of samples. It should be useful in the pharmaceutical industry for this important determination.

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Communications

Nonclassical Antimetabolites XVI. The Bridge Principle of Specificity with Active Site-Directed Irreversible Inhibitors, VII. Detection of Differences in Specificity of Enzymic Nucleophilic Sites by the Maleamyl Group

Sir:

The concept of (1) a new class of irreversible inhibitors that operate by active site-direction (exo-alkylation)[1] has been supported by strong experimental evidence (2); a properly designed inhibitor such as 4-(iodoacetamido)salicylic acid (I) can reversibly complex with the active site of an enzyme such as GDH,[2] then become irreversibly bound to the enzyme adjacent to the complexing site by an anchimerically assisted type of alkylation. Four other laboratories have subsequently and independently made related observations pertinent to this type of irreversible inhibition in the field of esterases (3–5) and in the field of immunochemistry (6). Our detailed version of the experimental evidence for active site-directed irreversible inhibition (7) led to the proposal of the bridge hypothesis of specificity:

Compared to a reversible inhibitor, the active site-directed type of irreversible inhibitor can have an extra dimension of specificity; this extra specificity is dependent upon the ability of the reversibly-bound inhibitor to bridge to and covalently link to a nucleophilic group on the enzyme surface and upon the nucleophilicity of the enzymic group being attacked.

Experimental evidence for the first corollary of the bridge hypothesis of specificity, namely, the difference in ability of certain reversibly bound inhibitors to bridge to and alkylate on enzymic nucleophilic site has been presented (8, 9). More recently experimental evidence for the second corollary, the difference in nucleophilicity of the enzymic groups being covalently linked, was demonstrated with compounds related to 5-(carbophenoxyamino)salicylic acid, whereby GDH was irreversibly inhibited, but LDH was not (10). In fact, the substrate-

TABLE I.—IRREVERSIBLE INHIBITION OF LDH BY 4-(MALEAMYL)SALICYLIC ACID (II)

Compound	I_{50}[c,d] GDH	I_{50}[c,d] LDH	Rate of Inactivation[f] GDH	Rate of Inactivation[f] LDH
I	3.4[e]	6.6[e]	1.0[g]	1.0[g]
II[a]	17[i]	7.3[i]	0[h]	0.9
III[b]	...	32[i]	...	0[h]

[a] Prepared in 96% yield from maleic anhydride and 4-aminosalicylic acid in boiling methyl ethyl ketone; light yellow crystals, m. p. 195–197° dec.; *Anal.*—Calcd. for $C_{11}H_9NO_6$: C, 52.6; H, 3.58; N, 5.57. Found: C, 52.7; H, 3.75; N, 5.68. [b] *Org. Syn.*, 41, 93(1961). [c] The I_{50} is defined as the concentration of inhibitor necessary to give 50% reversible inhibition in the presence of 1 millimolar of substrate. [d] 1 Millimolar α-oxoglutarate to L-glutamate or 1 millimolar pyruvate to L-lactate as previously described (9, 13). [e] From reference 9. [f] Rate of inactivation by 2 m*M* concentration of inhibitor was determined as previously described (7, 12). [g] Arbitrary value for comparison down the column; the absolute rates are different for the two enzymes (7). [h] Showed protection against thermal inactivation of the enzyme in some runs. [i] Estimated from the amount of inhibition obtained up to a 3 m*M* solution, the maximum concentration still permitting full light transmission. *i* Estimated from the amount of inhibition obtained up to a 16 m*M* solution, the maximum concentration permitting full light transmission.

[1] The term "active site-directed irreversible inhibition" is preferable to the term "exo-alkylation" used in previous papers since, first, the term is more self-explanatory and, second, some of the irreversible inhibitors operate by acylation or Michael addition and not by alkylation.

[2] Abbreviations: LDH, lactic dehydrogenase from rabbit skeletal muscle; GDH, glutamic dehydrogenase from mammalian liver.

SUMMARY

A procedure has been described for the spectrophotometric determination of the zinc content of various lots of insulin. This technique for quality control involves differential demasking as a means of precluding the effect of contaminating trace metals such as copper and iron. The process is simple, accurate, and capable of rapid analysis for large numbers of samples. It should be useful in the pharmaceutical industry for this important determination.

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Communications

Nonclassical Antimetabolites XVI. The Bridge Principle of Specificity with Active Site-Directed Irreversible Inhibitors, VII. Detection of Differences in Specificity of Enzymic Nucleophilic Sites by the Maleamyl Group

Sir:

The concept of (1) a new class of irreversible inhibitors that operate by active site-direction (exo-alkylation)[1] has been supported by strong experimental evidence (2); a properly designed inhibitor such as 4-(iodoacetamido)salicylic acid (I) can reversibly complex with the active site of an enzyme such as GDH,[2] then become irreversibly bound to the enzyme adjacent to the complexing site by an anchimerically assisted type of alkylation. Four other laboratories have subsequently and independently made related observations pertinent to this type of irreversible inhibition in the field of esterases (3–5) and in the field of immunochemistry (6). Our detailed version of the experimental evidence for active site-directed irreversible inhibition (7) led to the proposal of the bridge hypothesis of specificity:

Compared to a reversible inhibitor, the active site-directed type of irreversible inhibitor can have an extra dimension of specificity; this extra specificity is dependent upon the ability of the reversibly-bound inhibitor to bridge to and covalently link to a nucleophilic group on the enzyme surface and upon the nucleophilicity of the enzymic group being attacked.

Experimental evidence for the first corollary of the bridge hypothesis of specificity, namely, the difference in ability of certain reversibly bound inhibitors to bridge to and alkylate on enzymic nucleophilic site has been presented (8, 9). More recently experimental evidence for the second corollary, the difference in nucleophilicity of the enzymic groups being covalently linked, was demonstrated with compounds related to 5-(carbophenoxyamino)salicylic acid, whereby GDH was irreversibly inhibited, but LDH was not (10). In fact, the substrate-

TABLE I.—IRREVERSIBLE INHIBITION OF LDH BY 4-(MALEAMYL)SALICYLIC ACID (II)

Compound	I_{50}[c,d] GDH	I_{50}[c,d] LDH	Rate of Inactivation[f] GDH	Rate of Inactivation[f] LDH
I	3.4[e]	6.6[e]	1.0[g]	1.0[g]
II[a]	17[i]	7.3[i]	0[h]	0.9
III[b]	...	32[i]	...	0[h]

[a] Prepared in 96% yield from maleic anhydride and 4-aminosalicylic acid in boiling methyl ethyl ketone; light yellow crystals, m. p. 195–197° dec.; *Anal.*—Calcd. for $C_{11}H_9NO_6$: C, 52.6; H, 3.58; N, 5.57. Found: C, 52.7; H, 3.75; N, 5.68. [b] *Org. Syn.*, 41, 93(1961). [c] The I_{50} is defined as the concentration of inhibitor necessary to give 50% reversible inhibition in the presence of 1 millimolar of substrate. [d] 1 Millimolar α-oxoglutarate to L-glutamate or 1 millimolar pyruvate to L-lactate as previously described (9, 13). [e] From reference 9. [f] Rate of inactivation by 2 m*M* concentration of inhibitor was determined as previously described (7, 12). [g] Arbitrary value for comparison down the column; the absolute rates are different for the two enzymes (7). [h] Showed protection against thermal inactivation of the enzyme in some runs. [i] Estimated from the amount of inhibition obtained up to a 3 m*M* solution, the maximum concentration still permitting full light transmission. *i* Estimated from the amount of inhibition obtained up to a 16 m*M* solution, the maximum concentration permitting full light transmission.

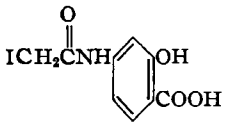
[1] The term "active site-directed irreversible inhibition" is preferable to the term "exo-alkylation" used in previous papers since, first, the term is more self-explanatory and, second, some of the irreversible inhibitors operate by acylation or Michael addition and not by alkylation.

[2] Abbreviations: LDH, lactic dehydrogenase from rabbit skeletal muscle; GDH, glutamic dehydrogenase from mammalian liver.

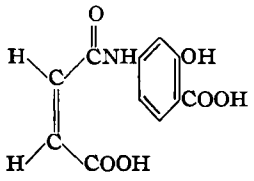
identical enzyme, LDH, from two different tissues could be irreversibly inhibited selectively by use of both corollaries (11, 12).

The fact that 4-(iodoacetamido)salicylic acid (I) could irreversibly inhibit both GDH and LDH was attributed to the low functional specificity of the iodoacetamido group. In contrast, 5-(carbophenoxyamino)salicylic acid irreversibly inhibited GDH, but not LDH; this selectivity was attributed to the specificity of the carbophenoxy group for reaction with a primary amino group which apparently was properly juxtapositioned in GDH to the carbophenoxy function for reaction, but was not in LDH. This paper describes a cross-over of functional specificity with 4-(maleamyl)salicylic acid (II).

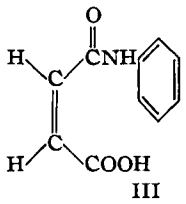
In Table I is listed a comparison[3] of both reversible and irreversible inhibition of GDH



I



II



III

and LDH by compounds I–III. Although 4-(maleamyl)salicylic acid (II) could reversibly bind to both enzymes, *only LDH was irreversibly* inhibited. Maleanilic acid (III) showed relatively weak reversible binding to LDH,[4] *but showed no irreversible inhibition.* The irreversible inhibition of LDH shown by II and not III is strong evidence that a properly oriented complex with the active site is an obligatory intermediate for irreversible inhibition; if II had inactivated LDH by random bimolecular reaction, then maleanilic acid (III) should have inactivated LDH even more rapidly than II, since II would protect the active site against bimolecular reaction (12). Bimolecular inactivation of enzymes by N-ethylmaleimide is a well known phenomenon, and maleanic acid also inactivates enzymes but at a much slower rate (14); since III does not inactivate LDH, the inactivation of LDH by II definitely operates by a different mechanism, presumably by active site-directed irreversible inhibition.

The α,β-unsaturated carbonyl system of II, maleimides, and maleamic acids react in general most rapidly with SH groups, much slower with amino groups, and extremely slowly with hydroxyl groups. Thus, it is probable that LDH has a properly juxtapositioned SH group within the LDH–II complex that allows for an anchimerically assisted Michael addition reaction, whereas GDH does not; in contrast, GDH is irreversibly inhibited by active site-directed acylation with the amine reagent 5-(carbophenoxyamino)salicylic acid, but LDH is not irreversibly inhibited by this reagent (10).

Further search for groups on a reversible inhibitor that can specifically bridge to and covalently link other enzymic functional groups is continuing; such group specific active site-directed irreversible inhibitors would be of use in both chemotherapy and protein structure studies.

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[3] The technical assistance of Dorothy Ackerman and Maureen Vince in making these measurements is acknowledged.

[4] III probably binds reversibly *via* the carboxylate in the fashion of benzoate and cinnamate (13), thus giving a conformationally different complex than I and II.

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— *The Editor comments* —

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Review Article

Use of Nonaqueous Solvents in Parenteral Products

By A. J. SPIEGEL and M. M. NOSEWORTHY

THE PRACTICE OF incorporating naturally occurring nonaqueous solvents such as fixed oils and glycerin in pharmaceuticals has been common for many years. Water is always the solvent of choice. However, when it is not possible for physical or chemical reasons (such as limited solubility and hydrolytic reactions) to use a wholly aqueous system, nonaqueous solvents aid the formulator in developing stable, convenient parenteral dosage forms. A parenteral solution avoids the disadvantages inherent in suspensions, such as nonuniform dosage, caking, and possible slow release of the medicament when it is not desired.

A formulator encounters many problems once he determines that an aqueous system is unsatisfactory. The chosen solvent must be nontoxic, nonirritating, and nonsensitizing. It also must exert no pharmacologic activity of its own, nor adversely affect the action of the medicament. There are reported instances in which a solvent potentiated the activity of the medicament necessitating a change of dosage level. This will be discussed in greater detail later in this review.

In addition to being pharmacologically acceptable, the chemical and physical properties of the solvent must be taken into account. Thus, the ideal solvent should not be affected by acids or alkalies and it should be generally stable under normal conditions of pharmaceutical use. The viscosity must be such as to allow for ease of injection, and the solvent must remain fluid over a fairly wide temperature range. It is advantageous if the solvent has a sufficiently high boiling point to allow heat sterilization. Additional considerations are water and body fluid miscibility, the degree of flammability, availability, source of supply, and constant purity.

Obviously, no such individual solvent presently exists. Thus, the selection of a nonaqueous solvent for a parenteral vehicle is a compromise among the many influencing factors. The advent of modern chemical technology has produced many new synthetic solvents in addition to the naturally occurring ones.

This review presents the toxicity, chemical and physical properties, and applications of some of the more commonly used nonaqueous solvents, as well as some specialized and rarely used solvents in pharmaceutical formulations.

FIXED OILS

The U.S.P. (1) recognizes the use of fixed oils as parenteral vehicles. Fixed oils are mainly mixtures of esters of unsaturated fatty acids which are fluid at 20°. The fluidity is generally due to the presence of the oleic acid esters of glycerin. The most commonly used fixed oils are corn oil, cottonseed oil, peanut oil, and sesame oil (2). Castor oil and olive oil have been used occasionally. While the toxicities of vegetable oils are relatively low, some patients exhibit allergic reactions to specific vegetable oils. Therefore, when such oils are used as vehicles, the label must state the specific oil contained in the product. Fixed oils have been known to cause undesirable

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local tissue reactions, such as cysts, foreign-body granulomas and, occasionally, nerve injury.

The requirements for fixed oils to be used in parenteral products are specified in the U.S.P. (1). The use of fixed oils is limited because of the low solubility of most drugs in these solvents. Since these oils are not miscible with water, dissolved drugs may exhibit a sustained-release effect with a possible diminution of absorption. Aqueous insolubility also precludes the use of fixed oils in unemulsified intravenous products.

Cottonseed oil has been used in intravenous fat emulsions administered to surgical patients as reported by Lehr and co-workers (3) and a commerical product is available.

Drugs which are incorporated in oils are mainly the steroid hormones, dimercaprol, calciferol, and menadione. Since fixed oils contain unsaturated fatty acids, oxidative changes take place which may necessitate the use of oil soluble antioxidants such as propyl gallate, butylated hydroxyanisole, butylated hydroxytoluene, and tocopherols.

A list of the official U.S.P. (1) and N.F. (4) parenteral solutions using fixed oils as solvents is given in Table I.

TABLE I.—OFFICIAL INJECTIONS IN OIL

Desoxycorticosterone acetate U.S.P.
Dimercaprol U.S.P.
Estradiol benzoate U.S.P.
Estradiol cyclopentylpropionate N.F.
Estradiol dipropionate U.S.P.
Estrone U.S.P.
Progesterone U.S.P.
Testosterone propionate U.S.P.
Diethylstilbestrol dipropionate N.F.
Menadione N.F.

A typical intramuscular formula for a testosterone propionate oil solution, 50 mg./ml., is (5)

	Gm./1000 ml.
Testosterone propionate	50.0
Benzyl alcohol	21.0
Sesame oil	869.0

Since sesame oil becomes turbid on cooling, a "winterized" or treated oil should be used, so that the oil remains clear when cooled to 5°.

ETHYL OLEATE

The "British Pharmacopoeia" (6) recognizes ethyl oleate as an alternative vehicle in injections of deoxycortone acetate, estradiol monobenzoate, progesterone, and testosterone propionate.

It is a yellowish oily liquid which is insoluble in water and miscible with alcohol, ether, and fixed oils. It has properties similar to fixed oils, except that it is less viscous, is a superior solvent, and is more rapidly absorbed by the tissues (7). Unlike untreated sesame oil, ethyl oleate remains clear at 5°, but it has the disadvantage of discoloring on standing.

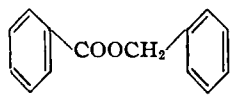
There are indications of increased hormone activity when ethyl oleate is used in place of sesame oil as a parenteral hormone vehicle. Studies by Dekanski and Chapman (8) demonstrated improved intensity and duration of action of testosterone phenylpropionate and testosterone propionate in ethyl oleate over that of the same androgens in sesame oil.

ISOPROPYL MYRISTATE

The use of isopropyl myristate as a vehicle for parenteral injections has been reported by Platcow and Voss (9). It is an oil miscible, water immiscible, chemically stable substance, not susceptible to rancidity and having a specific gravity of 0.852 (10). It consists mainly of isopropyl myristate and a small amount of isopropyl esters of other saturated fatty acids. Acute toxicity studies indicate a very low order of toxicity, but attempts to establish an LD_{50} in mice failed when dosages equivalent to 100 ml./Kg. did not affect the test animals. Isopropyl myristate shows a very low degree of irritability and exhibits no sensitizing properties in rabbits and guinea pigs following topical and parenteral administration. In experiments on ovariectomized rats it compared favorably with sesame oil as a repository vehicle for estrogens (9). The external pharmaceutical use has been evaluated by Donovan, *et al.* (11), who found it a useful intermediate solvent for the incorporation of phenol, cocaine, resorcinol, and salicylic acid into liquid petrolatum.

BENZYL BENZOATE

Benzyl benzoate (12) is a colorless, oily liquid with a pleasant aromatic odor. It has a specific gravity of 1.118, boils at 323°, and is insoluble in water or glycerin, but is miscible with alcohol, chloroform, ether, and fixed oils. Its structural formula is

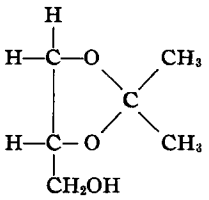


Benzyl benzoate has found some use as a cosolvent in oleaginous injectables such as dimercaprol injection, and in commercial preparations of hydroxyprogesterone benzoate where it is present in concentrations of 30% for the 125-mg. product in sesame oil, and 46% for the 250-mg.

product in castor oil. The formula for the injection of dimercaprol B.P. (1958) is dimercaprol 5.0 Gm., benzyl benzoate 9.6 ml., and peanut oil, to make 100 ml.

DIOXOLANES

The dioxolanes represent a new and interesting group of synthetic solvents for pharmacists. These substances are the reaction products of glycerin with ketones in the presence of a dehydrating agent (13). The least toxic member of the group is 2,2-dimethyl-1,3-dioxolane-4-methanol (14). This structural formula is



This compound [also known as Solketal, isopropylidene glycerol, and glycerol dimethylketal (15)] is reported to be a nontoxic, nonirritating solvent, miscible with water, alcohol, esters, aliphatic and aromatic hydrocarbons, and virtually all other organic solvents. It is a water-white, practically odorless liquid of medium viscosity, stable on storage, and unaffected by alkalies. Its boiling point is 82–83°, and has a specific gravity of 1.064. It is, however, hydrolyzed by strong aqueous acid solutions to acetone and glycerin (15–19).

Berger (14) reported the dioxolanes (as a class) produce transient muscular relaxation and paralysis. These effects were due to a depressant action on the central nervous system and not to a peripheral curare-like action. The mean paralyzing dose, ED_{50}, and the mean lethal dose, LD_{50}, for 2,2-dimethyl-1,3-dioxolane-4-methanol after intraperitoneal administration in mice was reported to be greater than 2.112 Gm./Kg. (16.0 mM/Kg.).

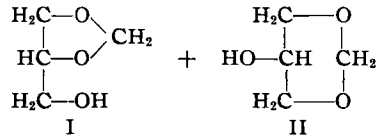
Teuber (20, 21) reported that the oral LD_{50} in mice is 4.0 to 7.2 Gm./Kg. (30–55 mM/Kg.). Dermatologic tests on rabbits produced no untoward reactions after 3 weeks of application.

Guinea pigs exposed to aerosol sprays for 1 hour a day for 3 days exhibited no inflammation of mucous membranes. Huber (22) reported on the use of antibiotic aerosol sprays utilizing this solvent as a carrier for penicillin and oxytetracycline.

In this country, 2,2-dimethyl-1,3-dioxolane-4-methanol has been reported in the patent literature as a water-miscible solvent in gelatin capsules (23) and as a parenteral vehicle for a tetracycline preparation (24).

GLYCEROL FORMAL

Glycerol formal is a condensation product of glycerol and formaldehyde consisting of 3-hydroxymethyl-1,3-dioxolane (I) (25%) and 5-hydroxy-dioxane (II) (75%). The structural formulas are

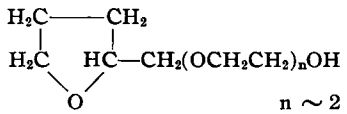


I II

It is a chemically stable, colorless, odorless liquid of low viscosity and is miscible with water in all proportions. Sanderson (25) reported that the LD_{100} for rats by intraperitoneal administration was 3000 mg./Kg. and that the maximum symptomless dose was 1500 mg./Kg. The use of glycerol formal as a nontoxic solvent in toxicity testing has been suggested. It has been used as an industrial solvent and no toxic effects have been reported (25).

GLYCOFUROL

Glycofurol is a Hoffmann-LaRoche trade name for a tetrahydrofurfuryl alcohol polyethylene glycol ether containing an average of two ethylene glycol units per molecule. It is a colorless liquid, miscible with water in all proportions and soluble in methanol, ethanol, *n*-propanol, and glycerin. It has a boiling point of 80–155° and a specific gravity of 1.078. The structure is (26)

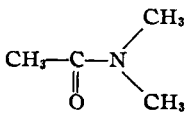


Spiegelberg and co-workers (26) extensively studied the pharmacology of this material and reported on its use as a parenteral solvent. Irritating when administered undiluted, it is nontoxic and nonirritating when diluted with water. The intravenous LD_{50} in the mouse is 3.78 Gm. (3.5 ml.)/Kg., and its tolerability equals that of propylene glycol. Acetylcholine chloride is reported to be stable in glycofurol solutions, while it is not stable in propylene glycol.

DIMETHYLACETAMIDE

Dimethylacetamide (DMA), also known as acetic acid dimethylamide and acetyldimethylamine, is an interesting solvent which warrants some discussion. It is a clear neutral liquid

having a boiling point of 165.5°, a specific gravity of 0.943, and a molecular weight of 87.12. The structural formula is



This solvent is miscible in all proportions with water and alcohol and very soluble in organic solvents and mineral oil (27).

Davis and Jenner (28) studied the acute toxicities of dimethylacetamide, dimethylformamide (DMF), and propylene glycol after single doses were administered intraperitoneally to mice. A 50% solution of DMA was used; however, the toxicity results are for the DMA content of the solution. The DMF and propylene glycol were administered undiluted. These results are as follows: the LD_{50} for DMF was 1122 mg./Kg., for DMA 3236 mg./Kg., and for propylene glycol 11,400 mg./Kg. The LD_{100} for DMA is 5012 mg./Kg.

Horn (29) investigated the chronic toxicity of DMA by dermal application in dogs at dosage levels of 0.1 to 4.0 mg./Kg. and by exposing both rats and dogs to an atmosphere containing DMA at concentrations of 40.0, 64.4, 102, and 195 p.p.m. All experiments were of 6-month duration unless obvious toxicity occurred. Liver damage occurred at all levels greater than 0.1 ml./Kg. dermally and 40 p.p.m. by inhalation.

The patent literature mentions the use of a 50% DMA solution as a vehicle for a preconstituted oxytetracycline (30) solution and as a solvent in soft and hard gelatin capsules (31). Its use as an anti-inflammatory agent in topical formulations is also reported (32).

Hammer, *et al.* (33), reported on a preconstituted intramuscular solution of oxytetracycline which consisted of a solution of an ethanolammonium magnesium salt of oxytetracycline in 50% N,N-dimethylacetamide. After testing in animals and humans, this formulation was found to be well tolerated and produced effective antibiotic serum levels. The stability was satisfactory for 2 years at room temperature.

A 50% solution of DMA is used as a solvent for a 250-mg./ml. chloramphenicol intravenous formulation, but it must be diluted with normal saline or 5% dextrose before administration.

A commercially available reserpine intramuscular product contains 10% DMA as a cosolvent (34).

DMA, when used as a drug solvent and administered to 15 patients with advanced malignancies produced hallucinations when given at levels above 400 mg./Kg. of body weight per day for 3 days or more (35). However, the normal parenteral level for DMA is equivalent to 30 mg./Kg. per day. Thus, in normal use this hallucinagenic effect would not be expected.

An oxytetracycline 50-mg./ml. formula (30) was reported to have been composed of oxytetracycline, 50 mg.; magnesium chloride, hexahydrate 1.7%; ethanolamine, 20% aqueous 1.5%; sodium formaldehyde sulfoxylate 0.2%; lidocaine 2%; and N,N-dimethylacetamide 50%, to make 1.00 ml.

N-(β-HYDROXYETHYL)-LACTAMIDE

N - (β - Hydroxyethyl) - lactamide (36), also known as lactic acid carboxamide, is a clear, colorless, syrupy liquid which is water miscible. The specific gravity of the pure compound is 1.192. It is used as a 50% solution and has the following formula: $CH_3CHOHCONHCH_2CH_2OH$. This compound is the reaction product of methyl acetate and 2-aminoethanol. The acute subcutaneous, LD_{50}, toxicity (37) for a 50% w/v N-(β-hydroxyethyl)-lactamide solution is 15.8 Gm. lactamide/Kg. in mice and 16.1 Gm. lactamide/Kg. in rats. This compound has been used in Europe as a solvent for a preconstituted oxytetracycline solution. Neumann (37) reported that this product was stable for several years and showed improved tissue tolerability.

Dimmling (38) has also reported on the use of N-(β-hydroxyethyl)-lactamide as a solvent for oxytetracycline. After 24 hours, a detectable serum level was found after a single dose of 250 mg. in ten healthy persons. Following a second injection of 250 mg. after an interval of 24 hours, the levels showed a cumulative increase. Further studies (39) on the serum concentrations confirmed the previous findings.

The results of Seeliger's (40) investigation with oxytetracycline intramuscular in N-(β-hydroxyethyl)-lactamide solution in patients have confirmed the previous values obtained in healthy individuals. A single injection of 250 mg. gave an effective serum concentration for over 24 hours. Following repeated injections on consecutive days, marked cumulative effects were observed. The clinical effect was in accordance with blood level determinations. Survey of local tolerability showed practically no pain in 93.7% of the 380 injections performed; slight and tolerable local reactions, which in no case persisted for more than 2 or 3 hours, were found in 6.3%.

Hupe (41) reported that in 90 major surgical cases 250 mg. of oxytetracycline intramuscular in this solvent, once a day, was effective and well tolerated.

The patent literature also refers to the use of N-(β-hydroxyethyl)-lactamide as a solvent for oxytetracycline injectables (30, 42). The use of other alkylol amides such as the amides of β-hydroxybutyric acid, succinic acid, adipic acid, tartaric acid, glycolic acid, and salicylic acid, was also mentioned (42). A typical formula for a 250 mg./3 ml. oxtyetracycline product is

	Gm./100 ml.
Oxytetracycline hydrochloride	9.62
Magnesium chloride-hexahydrate	4.00
Sodium formaldehyde sulfoxylate	0.20
Water, pyrogen-free	44.20
N-(β-hydroxyethyl)-lactamide	50.00
Monoethanolamine	2.30

ETHYL LACTATE

Ethyl lactate, ethyl α-hydroxypropionate, $CH_3CH(OH)COOCH_2CH_3$, is a colorless liquid with a specific gravity of 1.042 which is miscible with water and has a characteristic odor. In aqueous solution some decomposition takes place (43).

Latven and Molitor (44) determined the acute toxicity of ethyl lactate in mice by subcutaneous and intravenous administration, and their results are shown in Table II.

TABLE II.—ACUTE TOXICITY OF ETHYL LACTATE

	LD_0	LD_{50}	LD_{100}	Minimum Sympt. Dose	Maximum Nonsympt. Dose
Subcutaneous, ml./Kg.	2.0	2.5	3.0	1.0	0.8
Intravenous, ml./Kg.	0.2	0.6	1.0	0.3	0.2

Ethyl lactate was irritating on intradermal injection in guinea pigs and on application to the eyes of rabbits.

Ethyl lactate (10–100%) solubilizes an esterone injection in castor oil to a concentration of 3.5 to 6.5 mg./ml. (45). This product is stable at room temperature (46). It has been used as an industrial solvent and no toxic effects from its use have been recorded (47).

ETHYL CARBONATE

Ethyl carbonate, diethyl carbonate, $CH_3CH_2OCOOCH_2CH_3$, has also been used as a solvent for erythromycin, but there is a paucity of literature on its use and toxicity. It is a liquid immiscible with water but miscible with alcohol and ether and has a specific gravity of 0.975 and a boiling point of 126° (48). This compound has also been used as an industrial solvent with no reported toxic effects (49).

POLYETHYLENE GLYCOLS

The polyethylene glycols (PEGs), as the name implies, are polymers of ethylene oxide (50) with the general formula: $HOCH_2(CH_2OCH_2)_nCH_2OH$, where n represents the average number of ethylene oxide groups. These polymeric products are designated by a number which represents the average molecular weight (51). Polyethylene glycol 200, 300, 400, and 600 are moderately viscous, colorless, somewhat hygroscopic liquids. They are less volatile than glycerin and do not hydrolyze or deteriorate (52). They dissolve in water in all proportions to form clear solutions (51). Polyethylene glycol 1000, 1540, 4000, and 6000 are white waxy solids (Table III).

For the purpose of this discussion, we will confine our comments to the liquid polyethylene glycols which are more likely to be used in parenteral products. The literature abounds with papers discussing and describing measurements of the toxicity of the various PEGs by oral or topical routes (50–58). However, there is a scarcity of material on the parenteral administration. PEG 300 and 400 are better described than the other two members of the liquid PEG series. Only the parenteral LD_{50}'s (as shown in Table IV) were found by the authors. Oral and dermal toxicity data are given in Tables V and VI.

Subcutaneous dosages of PEG 400 up to 10 ml. (ten times the human dose) in rats caused no permanent damage. The reactions were described as "blanching of the skin and scab formation in 48 hours." The test results were reported to be the same as with propylene glycol. PEG 300 and 400 do not elicit a foreign body reaction in animals (52). In dogs, the removal of PEG

TABLE III.—PHYSICAL PROPERTIES OF POLYETHYLENE GLYCOLS (51)

PEG	Av. Mol. Wt.	Sp. Gr., 20° C.	Freezing or Melting Range, ° C.	Viscosity, Cps. at 210° F.	Comparative Hygroscopicity Glycerol = 100
200	190–210	1.125	Super Cools	4.3	70
300	285–315	1.125	−15 to −8	5.8	60
400	380–420	1.125	4 to 8	7.3	55
600	570–630	...	20 to 25	10.5	40
1000	950–1050	1.151	37 to 40	17.4	35
1540	1300–1600	1.151	43 to 46	25–32	30
4000	3000–3700	1.204	53 to 56	75–85	...
6000	6000–7500	...	60 to 63	700–900	...

TABLE IV.—PARENTERAL TOXICITY OF POLYETHYLENE GLYCOLS

PEG	Animal	Route	Dose	Value, mg./Kg.	Ref.
300	Female rat	i.v.	LD_{50}	7,979	52
300	Rat	i.p.	LD_{50}	19,125	50
400	Mouse	i.p.	LD_{50}	4,200	59

TABLE V.—ORAL AND DERMAL TOXICITY OF POLYETHYLENE GLYCOLS IN MICE (60)

PEG	LD_{50} Mouse, oral ml./Kg.	Sublethal Dose Mouse, oral ml./Kg.	Sublethal Dose Mouse, s.c. ml./Kg.	LD_{100} Mouse, oral ml./Kg.
200	38.3	30.0	6.5	55
600	47.0	16.0	8.0	60

TABLE VI.—ORAL TOXICITY OF POLYETHYLENE GLYCOLS IN RATS (51)

Polyethylene Glycol	Single Oral LD_{50} Rats Gm./Kg.	No-Effect Dose with Repeated Feedings, Rats Gm./Kg./Day
200	28.9 ml. (32.51 Gm.)	0.88 (2 yr.)
300	31.7 ml. (35.66 Gm.)	5.4 (90 days)
400	43.6 ml. (49.05 Gm.)	0.96 (2 yr.)
600	38.1 ml. (42.86 Gm.)	2.42 (90 days)

from the site of injection is rapid, since the material diffuses freely into surrounding tissue.

When PEG 400 was injected intravenously into humans, 77% was recovered in the first 12 hours. Intramuscular injections in rats of five to ten times the expected human dosage levels of PEG 300 produced ischemic necrosis of the muscle fibers when the dose infiltrated a muscle bundle. The tissue response was one of mild chemical inflammation (50).

Lee and Anderson (61) determined the toxicity of vancomycin in 50% PEG 200 and of PEG 200 alone. Their results indicated that PEG 200 produced no apparent toxic effects when given to dogs at 1.0 ml./Kg. per day for 80 days intramuscularly, or of 0.5, 1.0, 2.5, and 5.0 ml./Kg. as single intravenous doses. Venous carbon dioxide content, blood nonprotein-nitrogen, and blood alkaline phosphatase were normal, no gross or microscopic abnormality was found in the kidneys, circulatory system, or other organs.

A dosage form for intravenous use containing nitrofurantoin was marketed some years ago containing PEG 300. In a study by McCabe, *et al.* (62), it was found that daily administration of 240 mg. of nitrofurantoin in PEG 300 to 30 patients caused severe metabolic acidosis and nephropathy in seven resulting in two deaths. These damaging effects were attributed to the PEG rather than nitrofurantoin and that dosage form was withdrawn from the market.

It should be noted that drugs dissolved in the PEGs may well present a toxicity and drug level much different from those reported in aqueous solutions or suspensions (51).

Swanson and co-workers (63) investigated the effect on the activity and toxicity of sodium amobarbital and sodium secobarbital by the addition of 60 and 70%, respectively, of polyethylene glycol 200. Their findings indicated that the addition of PEG 200 showed approximately the same potency and toxicity as aqueous solutions of these barbiturates. The median anesthetic dose (AD_{50}) by vein in rats was 75.0 ±3.5 mg./Kg. for sodium amobarbital and 39.0 ±1.2 mg./Kg. for sodium secobarbital. The median lethal doses (LD_{50}) were approximately twice as large as the AD_{50}'s. This ratio of nearly 2:1 is common to most barbiturates in use. It was also reported that subacute toxicity experiments show that both barbiturates in PEG 200 produced no obvious pathological changes. When injected intramuscularly in rabbits the aqueous solutions of the two barbiturates produced more irritation in tissues than solutions in PEG 200.

Bodin and Taub (64) investigated the stability of sodium pentobarbital in aqueous solutions containing 0 to 60% of polyethylene glycol 400. Their results indicated that the pH influences the concentration of polyethylene glycol 400 required for optimum stability. At a concentration of 30% and at a pH of 10, aseptic formulation of a stable product is possible. The addition of 10% ethanol permits sterilization by autoclaving without discoloration. It is also possible to prepare formulations containing 60% of the glycol and 10% ethanol at a pH as low as 8. These solutions are also stable to autoclaving.

Linde (65) studied the extent to which a number of sodium salts of 5,5-disubstituted barbiturates such as phenobarbital, barbital, aprobarbital, amobarbital, and pentobarbital could be stabilized by propylene glycol, polyethylene glycol 400, glycerin, and alcohol. Both the glycols, as well as alcohol, showed the same stabilizing activity. Glycerin had considerably lower stabilizing properties. With glycols as stabilizers, the various barbiturate derivatives studied displayed great differences in stability. The concentration of the solvent added to the solution was found to be the factor governing the stabilizing effect. The stability increased as the concentration was increased. A concentration of

50% was the highest used. The author (65) also established that stability of barbiturates dissolved in pure propylene glycol and polyethylene glycol 400 was very good, and that a lowering of pH in alcohol solutions decreases the rate of deterioration.

The use of 10% polyethylene glycol 300 as a solubilizing agent for a 2.5 mg./ml. injection of reserpine has been reported by Leyden, *et al.* (66). Commercial reserpine products containing 10–30% PEG 300, as well as 25% PEG 400, are available.

Higuchi and Lach (67) reported that although pentobarbital and barbital have little or no tendency to complex with polyethylene glycols, phenobarbital forms stable and stoichiometric molecular compounds with these macromolecular substances. Analysis indicates that one phenobarbital molecule is bound by two ethylene oxide units of the polyether chain. They have also shown that phenolic compounds are bound by the polyethers in the same manner, the higher molecular weight polymers exhibiting greater complexing tendency than those of lower degrees of polymerization. Several organic acids, such as salicylic acid and *p*-hydroxybenzoic acid, are only weakly bound.

An erythromycin ethyl succinate intramuscular product in a PEG vehicle is available, as is a secobarbital parenteral product in a 50% PEG vehicle. The specific PEG used was not indicated in either case.

GLYCERIN

Glycerin (68) is a clear, viscous, high-boiling liquid, which is miscible with water and alcohol and is a good solvent for many compounds. It is hygroscopic and will absorb several times its weight of water under conditions of high humidity. Glycerin tends to supercool rather than crystallize at lower temperature; its aqueous solutions resist freezing.

The toxicity (69) is low with oral administration. No deleterious effects were observed in man with 110 Gm. of glycerin per day for 50 days. Normal growth and reproduction occurred in rats with 41% of glycerin added to the food for 40 weeks, and in dogs with 35% added for 50 weeks. Drill (70) reports the oral LD_{50} in rats for undiluted glycerin to be 25 Gm./Kg. and the intravenous LD_{50} is 5 to 6 Gm./Kg.

There appears to be some controversy regarding the use of glycerin in parenterals, as indicated by reports that its administration to animals has caused hemoglobinurea (71, 72), hypotension (73, 74) and central nervous disturbances (74), and weight loss (75). Hanke (76) has given an excellent review of the physiologic action of glycerin.

There are, however, references to its use in human parenteral therapy. No toxic effects were noted after the administration of very small amounts of glycerin by intra-arterial administration in the treatment of elephantiasis (77).

Sloviter (78, 79) reported that the intravenous administration of solutions containing 5% glycerin to experimental animals and to man caused no toxic or other undesirable effects. Humans were given intravenously 50 Gm. of glycerin in a liter of solution which also contained 50 Gm. of glucose and 9.0 Gm. of sodium chloride. No disturbances of cardiorespiratory or central nervous system function occurred, and no significant hemolytic effects occurred. Sloviter (78, 79) further stated that the previously reported toxic effects of parenterally administered glycerin were not observed, and that there were indications that these toxic effects were due to the osmotic disturbances produced by the injection of solutions of high glycerin concentrations. In the dog, the rapid injection of a concentrated solution produced a transient drop in blood pressure which the author believed to be due to a peripheral vasodilating effect. It was also suggested that intravenously administered glycerol may be useful as a nutritional agent as well as for a solvent in preparations of drugs for intravenous administration (79).

Large parenteral doses cause convulsant and paralytic symptoms through direct action on the central nervous system. The blood corpuscles are also laked, probably caused by an osmotic effect as previously mentioned. The glycerin remains for a time, unabsorbed, and in high concentration at the site of injection, and the corpuscles are probably laked during their passage through this area (69).

Lachaux (80) states that aqueous solutions of up to 30% glycerin are well tolerated intramuscularly. Absorption is good and there is rapid dilution by the body fluids.

Latven and Molitor (44) determined the intravenous and subcutaneous toxicity in white mice. Their results are shown in Table VII.

TABLE VII.—ACUTE TOXICITY OF GLYCERIN

	LD_0	LD_{50}	LD_{100}	Min. Sympt. Dose	Max. Non-Sympt. Dose
Subcutaneous, ml./Kg.	8.0	10.0	12.0	8.0	7.0
Intravenous, ml./Kg.	5.0	6.0	7.0	3.0	2.0

Krause and Cross (81) have found that the solubility of phenobarbital in alcohol was enhanced by the addition of glycerin. The maximum solubility of phenobarbital in alcohol–glycerin mixtures was reached at a level of 80% alcohol and 20% glycerin.

Linde (65), in his work on sodium salts of barbiturates, found that glycerin has considerably lower stabilizing properties than propylene glycol, polyethylene glycol, and alcohol.

Husa and Jatul (82) found that after heating a sodium phenobarbital solution in 50% glycerin for 30 minutes at 115°, deterioration was 8% compared to 9% in pure water solution.

The U.S.P. (1) allows the use of glycerin in injections of deslanoside and digitoxin. A commercial product of deslanoside contains 15% glycerin.

ETHANOL

Ethanol (alcohol), ethyl alcohol, finds occasional use in parenteral products, particularly the digitalis glycosides. A commercial digitoxin preparation for intramuscular or intravenous use contains 49% alcohol. This produces pain on intramuscular administration. The U.S.P. (1) states that such a product can contain 5–50% alcohol. A digoxin preparation containing 10% alcohol, as allowed by the U.S.P., may be used for intramuscular or intravenous administration. A deslanoside product containing 7.4% alcohol is also available.

Alcohol injected subcutaneously causes considerable pain followed by anesthesia. If injection is made close to nerves, neuritis and nerve degeneration may occur. Injection in or near nerves is deliberately used to cause anesthesia in the treatment of severe pain. The intravenous anesthetic dose is 2–3 ml. of 95% alcohol/Kg. (83).

Latven and Molitor (44) have reported the LD_{50} in mice to be 1973 mg./Kg. intravenously, and 8285 mg./Kg. subcutaneously.

A commercial hydrocortisone intravenous product contains 50% alcohol. Mephensin injection B.P. (1958) contains 25% alcohol, its formula is mephenesin 10 Gm.; alcohol (95%) 25 ml.; propylene glycol 15 ml.; and water, pyrogen-free, to make 100 ml.

The formula for digoxin injection B.P. (1960 Supplement) is digoxin 25 mg.; alcohol (80%) 12.5 ml.; propylene glycol 40 ml.; citric acid 75 mg.; sodium phosphate 0.45 Gm.; and water, pyrogen-free to make 100 ml.

PROPYLENE GLYCOL

Seidenfeld and Hanzlik (84) in 1932 stated that propylene glycol, which was first described in 1895, has commanded little or no interest in chemistry or medicine even though it is available commercially. Today, it is one of the most widely used nonaqueous solvents.

Propylene glycol, 1,2-propanediol, is a viscous hygroscopic liquid with a specific gravity of 1.036. It freezes at −59° and boils at 188° and is miscible with water, acetone, and chloroform, but is immiscible with fixed oils. Under ordinary conditions it is very stable but at high temperatures it is oxidized to propionaldehyde, lactic acid, pyruvic acid, and acetic acid (85, 86).

The toxicity of propylene glycol has been extensively studied (44, 84, 87–91). Seidenfeld and Hanzlik (84) reported the intravenous minimum fatal dose for white rats to be 1.68 Gm./Kg. and for rabbits, 5.25 Gm./Kg. The intramuscular minimum fatal dose was 14.7 Gm./Kg. for rats and 7.5 Gm./Kg. for rabbits. Mice are more sensitive to propylene glycol (92) and have a lower toleration for this solvent. The LD_{50} for mice by intraperitoneal injection was found to be 9.7 Gm./Kg. (92). The subcutaneous and intravenous LD_{50} for mice was reported to be 18.5 Gm./Kg. and 8.0 mg./Kg., respectively (44). The action of propylene glycol on the central nervous system in dogs is similar to that of ethyl alcohol; however, its activity is about one-third that of ethyl alcohol (93).

Brittain and D'Arcy (94) reported that intravenous injections of propylene glycol in saline solution up to 50% in rabbits had no effect on the red blood cell count, packed cell volume, hemoglobin, or total white blood cell count. There was, however, an increase in the number of circulating polymorphs and a decrease in the lymphocytes. Blood clotting time was markedly decreased.

Heine, *et al.* (95), have given several formulas illustrating the use of propylene glycol in the preparation of barbiturate solutions for intramuscular or intravenous use

Sterile Solution of Sodium Pentobarbital 0.150 Gm./ml.	
Sodium pentobarbital	15.0 Gm.
Benzyl alcohol	2.0 ml.
Propylene glycol	60.0 ml.
Water for injection, to make	100.0 ml.

Sterile Solution of Sodium Amobarbital 0.250 Gm./ml.	
Sodium amobarbital	25.0 Gm.
Benzyl alcohol	2.0 ml.
Propylene glycol	60.0 ml.
Water for injection, to make	100.0 ml.

Sterile Solution of Sodium Phenobarbital 0.150 Gm./ml.	
Sodium phenobarbital	15.0 Gm.
Benzyl alcohol	2.0 ml.
Propylene glycol	60.0 ml.
Water for injection, to make	100.0 ml.

Brass (96) prepared a quinidine formulation for intramuscular use composed of 10.0 Gm. of quinidine hydrochloride and 75.0 ml. of propylene glycol. This solution showed no signs of discoloration or crystallization over a period of 6 months. When injected in man, the evidence of the action of quinidine is detectable in 15 minutes and persists for approximately 2 hours.

Gluck, *et al.* (97), tested a 20% solution of quinidine sulfate in propylene glycol for its local irritant effects by intramuscular injection and for its action on the auricle in auricular fibrillation and flutter. The results indicated negligible local reaction and a dose response similar to that obtained with oral administration—peak effect being reached in an average of about 3 hours, about half of the effect wearing off in about 8 hours, and the effect disappearing completely in 24 hours or less.

The use of propylene glycol as a vehicle for the intravenous administration of desoxycorticosterone acetate in a concentration of 10 mg./ml. has been discussed by McGavack and Vogel (98). Since this compound crystallizes out when diluted with water, a technique is reported whereby the preparation is injected slowly, not to exceed a rate of 2.5 ml. per minute. This assures its rapid dilution and prevents the formation of any acicular precipitate.

Ganz and co-workers (99) have studied the intramuscular administration of digoxin in 40% propylene glycol and 10% ethanol in patients with auricular fibrillation and reported satisfactory results.

The comprehensive use of propylene glycol is reviewed in articles by Parisi (100), Gialdi and Baruffini (101), Brown (102), and Heine, *et al.* (95).

Gershenfeld and Witlin (103) investigated the use of propylene glycol as a solvent for iodine and suggested that iodine 2% and sodium iodide 2.4% in distilled water containing 25 to 50% propylene glycol would be a suitable formula.

Linde (65) reported that the stability of barbiturates dissolved in pure or diluted propylene glycol was satisfactory.

Peterson and Hopponen (104) determined the solubility of phenobarbital in propylene glycol–alcohol-water systems. The solubility reached a maximum at a 50% mixture of propylene glycol and alcohol. Propylene glycol, 100%, dissolves more phenobarbital than 100% alcohol. Upon addition of water to the solvents, the solubility falls off more quickly with propylene glycol than with alcohol.

Propylene glycol was found to potentiate the preservative action of the parabens (105). Mehta and Drommond (106) determined the stability of various medicaments in propylene glycol after storage at 25°.

Marcus and Taraszka (107) reported that the specific hydrogen ion–catalyzed degradation of chloramphenicol in solutions containing up to 50% (v/v) of propylene glycol was qualitatively similar to degradation in purely aqueous systems. The reactions remain pseudo-first order with respect to chloramphenicol and the direct dependence of the rate upon acid concentration is maintained. Their results show that it is unwise to assume that replacement of all or a portion of the water in a vehicle will always enhance the stability of the active ingredient.

Huttenrauch (108) has shown that propylene glycol enhances the stability of L-ascorbic acid in tests run for 120 weeks at room temperature.

Weinstein, *et al.* (109), reported that oxytetracycline intramuscular solution in propylene glycol was well tolerated and produced prompt, prolonged, satisfactory blood levels. A 250 mg./-2 ml. oxytetracycline intramuscular formula would be

Oxytetracycline	250 mg.
Lidocaine	2%
Magnesium chloride, hexahydrate	6%
Sodium formaldehyde sulfoxylate	0.5%
Ethanolamine	4.2%
Propylene glycol	67%
Water	16.8%

This formula is stable for 2 years at room temperature. Without the use of propylene glycol, the stability is of 2-day duration only.

Hanson (110) compared the amount of tissue reaction produced by subcutaneous and intramuscular injections of a number of parenteral preparations of broad-spectrum antibiotics. The smallest amount of tissue reaction was produced by propylene glycol solutions of oxytetracycline.

1,3-BUTYLENE GLYCOL

1,3-Butylene glycol, 1,3-butanediol, is a colorless, viscous liquid having a specific gravity of 1.005, a boiling point of 204°, and is soluble in water and alcohol (111). It is possible to modify the effect of drugs by choosing the proper solubilizing agent. In this manner, we can cause an increase or decrease in the effect of a drug. Bornmann, *et al.* (112), demonstrated that 1,3-butylene glycol can prevent the toxic reactions of pentamethylenetetrazol. It was also shown that the action of morphine hydrochloride, meperidine hydrochloride, and methadone hydrochloride in 1,3-butylene glycol, as compared to aqueous solutions, gave stronger and more prolonged effects

in a shorter time. Thus, by the use of this solvent, the dose may be lowered and undesirable side effects eliminated.

Bornmann and Loeser (113) reported on the use of propylene glycol and 1,3-butylene glycol as solvents for meprobamate. The 1,3-butylene glycol preparation was found to be slightly more toxic than the propylene glycol product, but both were suitable as solvents for the drug.

Bornmann (114–116) in his review of glycol toxicity, stated that 1,3-butylene glycol had a slight acute as well as a slight chronic toxicity. The LD_{50} subcutaneously in mice is 16.51 ml./-Kg. and in rats is 20.06 ml./Kg.

SUMMARY

A large number of organic solvents are currently available to the pharmaceutical formulator The three most commonly used nonaqueous solvents are fixed oils, propylene glycol, and the polyethylene glycols. The remaining solvents discussed are of minor importance and are used only in specific instances.

The use of nonaqueous solvents in parenteral products offers greater latitude in the formulation of new dosage forms. Such solvents, however, should be used only if a definite need is established. It must be recognized that any formulation containing a nonaqueous solvent is potentially a new entity and must be adequately tested.

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Research Articles

Nonclassical Antimetabolites XII

Bridge Principle of Specificity with Exo-Alkylating Irreversible Inhibitors V. Differences in Specificity of Enzymic Nucleophilic Sites as Detected by the Carbophenoxy Group

By B. R. BAKER and R. P. PATEL

Investigation of four carbophenoxyamino derivatives of salicylic acid and oxanilic acid has shown that all four reversibly bind to lactic dehydrogenase (LDH) and glutamic dehydrogenase (GDH). Three of these compounds showed irreversible inhibition of GDH with no irreversible effect on LDH. Since 4-(iodoacetamido)-salicylic acid had previously been shown to inhibit both enzymes irreversibly, the specificity shown by the carbophenoxyamino group is attributed to its specificity for reaction only with a primary amino group in the nucleophilic site of an enzyme.

RELATIVELY LARGE molecules—compared to the substrate—have been found that can inhibit an enzyme and have accordingly been called nonclassical antimetabolites (3, 4), in contrast to classical antimetabolites that have only a small change in structure compared to the substrate. Based on the nonclassical antimetabolite theory, it was possible to propose the concept of irreversible inhibition by an exo-alkylation mechanism (3); a properly designed inhibitor such as 4-(iodoacetamido)salicylic acid can reversibly complex with glutamic dehydrogenase (GDH),[1] then become irreversibly bound within the complex by alkylation of the enzyme adjacent to the active site. Strong experimental evidence to support this exo-alkylation phenomenon has been presented (5, 6).

Experimental observations pertinent to the exo-alkylation phenomenon were subsequently

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A preliminary communication on part of this work has appeared (2).
For previous paper in this series see Reference 1.

[1] LDH, lactic dehydrogenase; GDH, glutamic dehydrogenase; DPNH, reduced diphosphopyridine nucleotide; Tris, tris-(hydroxymethyl)aminomethane hydrochloride buffer; 4-ISA, 4-(iodoacetamido)salicylic acid.

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Bridge Principle of Specificity with Exo-Alkylating Irreversible Inhibitors V. Differences in Specificity of Enzymic Nucleophilic Sites as Detected by the Carbophenoxy Group

By B. R. BAKER and R. P. PATEL

Investigation of four carbophenoxyamino derivatives of salicylic acid and oxanilic acid has shown that all four reversibly bind to lactic dehydrogenase (LDH) and glutamic dehydrogenase (GDH). Three of these compounds showed irreversible inhibition of GDH with no irreversible effect on LDH. Since 4-(iodoacetamido)-salicylic acid had previously been shown to inhibit both enzymes irreversibly, the specificity shown by the carbophenoxyamino group is attributed to its specificity for reaction only with a primary amino group in the nucleophilic site of an enzyme.

RELATIVELY LARGE molecules—compared to the substrate—have been found that can inhibit an enzyme and have accordingly been called nonclassical antimetabolites (3, 4), in contrast to classical antimetabolites that have only a small change in structure compared to the substrate. Based on the nonclassical antimetabolite theory, it was possible to propose the concept of irreversible inhibition by an exo-alkylation mechanism (3); a properly designed inhibitor such as 4-(iodoacetamido)salicylic acid can reversibly complex with glutamic dehydrogenase (GDH),[1] then become irreversibly bound within the complex by alkylation of the enzyme adjacent to the active site. Strong experimental evidence to support this exo-alkylation phenomenon has been presented (5, 6).

Experimental observations pertinent to the exo-alkylation phenomenon were subsequently

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A preliminary communication on part of this work has appeared (2).
For previous paper in this series see Reference 1.

[1] LDH, lactic dehydrogenase; GDH, glutamic dehydrogenase; DPNH, reduced diphosphopyridine nucleotide; Tris, tris-(hydroxymethyl)aminomethane hydrochloride buffer; 4-ISA, 4-(iodoacetamido)salicylic acid.

and independently made in two other laboratories. Lawson and Schramm (7) observed that the *p*-nitrophenyl ester of N-bromoacetyl-L-α-aminobutyric acid rapidly released *p*-nitrophenol when allowed to react with chymotrypsin; the resultant bromoacetyl-L-α-aminobutyryl derivative of chymotrypsin (presumably bound by an ester link to a serine residue) underwent intramolecular alkylation between the bromoacetyl group and one of the two methionine residues in chymotrypsin. Wofsy, Metzger, and Singer (8) have made related observations in the area of hapten immunochemistry.

In our detailed version of the experimental evidence for the exo-alkylation phenomenon (6), the bridge hypothesis of specificity was proposed. Compared to a reversible inhibitor, "the exo-alkylating type of irreversible inhibitor can have an extra dimension of specificity; this extra specificity is dependent upon the ability of the reversibly bound inhibitor to bridge to and alkylate a nucleophilic group on the enzyme surface and upon the nucleophilicity of the enzymic group being alkylated."

Recently we presented experimental evidence (9, 10) for the first corollary of the bridge hypothesis of specificity; that is, the difference in ability of certain inhibitors to bridge to and alkylate an enzymic nucleophilic site which merited raising the status of the hypothesis to principle. Evidence has now been found to support the second corollary of the bridge hypothesis —namely, the difference in nucleophilicity of the enzymic group being alkylated; this evidence is the subject of this paper.

MATERIALS AND METHODS

Reagents.—Sodium pyruvate, α-ketoglutaric acid, DPNH, LDH, and GDH were purchased from Nutritional Biochemical Corporation and from Sigma Chemical Co.; LDH was the crystalline enzyme isolated from rabbit muscle and GDH the crystalline enzyme isolated from mammalian liver.

Reversible Binding of Inhibitors to Enzymes (I_{50}).—The I_{50} is defined as the millimolar concentration of inhibitor necessary to give 50% inhibition in the presence of 1 m*M* concentration of the substrate (4). The procedure employed for LDH was the same as previously described, except that Tris buffer (pH 7.4) and 1 m*M* pyruvate were employed; the I_{50} values for GDH were determined in the same fashion as for LDH using cell concentrations of 1 m*M* α-ketoglutarate and 75 m*M* ammonium sulfate in Tris buffer (pH 7.4).

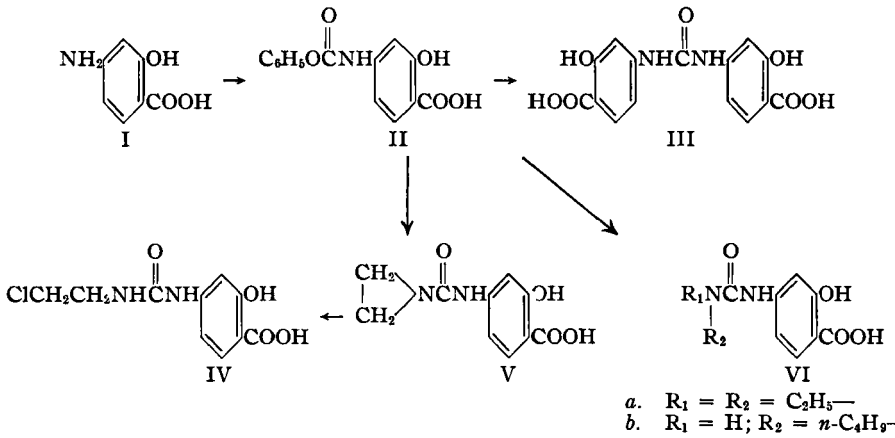
Enzyme Inactivation Procedure.—The LDH inactivation procedure (6), as later modified (10) was employed. The GDH incubations were run as previously described (6). In all incubations for determination of irreversible inhibition, three incubation solutions were run simultaneously and were made from the same master enzyme-DPNH solution (6): (*a*) a 2 m*M* concentration of test compound, (*b*) a standard with 2 m*M* 4-ISA, and (*c*) an enzyme control with no inhibitor. All runs were duplicated at least twice. An occasional bad run was readily eliminated if the control or standard did not behave properly.

The rate saturation experiments were run similarly, except that (*b*) was either a 1 m*M* solution or a 4 m*M* solution of the test compound depending upon the extent of reversible binding of inhibitor by the enzyme, that is, the I_{50}. Considerable care must be taken in making up solutions of the carbophenoxy compounds (Table II) so that the base labile phenyl ester group did not hydrolyze. Usually, 6 m*M* master solutions were made by stirring in Tris buffer at 0° with the gradual addition of 1 *N* sodium hydroxide to keep the pH at 7.4, but care was taken that the pH did not rise above 7.4; several hours were usually required to obtain complete solution. A 12.2 m*M* solution of the standard, 4-ISA, in pH 7.4 Tris buffer was stable for several weeks at 2–5°, as determined by the thiosulfate titration of covalently bound iodine (6). However, the carbophenoxy compounds as 6 m*M* solutions in Tris buffer (pH 7.4) were 25–30% hydrolyzed after 24 hours at 5° (determined spectrophotometrically) and thus were prepared fresh for each incubation.

CHEMISTRY

The synthesis of phenylurethanes and their further reaction with amines to give mixed ureas has been described by Crosby and Niemann (11). When 4-aminosalicylic acid (I) in aqueous sodium bicarbonate was treated under the usual conditions used for reaction with acid chlorides (12), 4-(carbophenoxyamino)salicylic acid (II) was obtained in low and variable yields and considerable amounts of the symmetrical urea (III) were formed.

The usual conditions (12) of dropwise addition of the acid chloride to a solution of I in aqueous sodium bicarbonate at near 0° were unsatisfactory because the initially formed product (II) in the alkaline solution could further react with as yet unreacted 4-aminosalicylic acid (I) to give the symmetrical urea (III). With amines soluble in an organic solvent, Crosby and Niemann (11) avoided this difficulty by reverse addition of the amine to the acid chloride; since the chloro group is more reactive than the phenoxy group, good yields of O-phenylurethanes could be obtained. Such a method would be incompatible with the aqueous alkaline method, since the acid chloride would merely undergo hydrolysis if insufficient amine were present. This enigma was resolved by adding the phenyl chloroformate all at once to the cold solution of I in aqueous sodium bicarbonate; this was possible since the chloroformate reacted slowly enough that the temperature was capable of being controlled. However, it is essential to have sufficiently vigorous stirring in this reaction to emulsify the acid chloride; magnetic stirring was insufficient and allowed the product (II) in solution to compete favorably for the 4-aminosalicylic acid (I), resulting in large amounts of the urea III being formed. The presence of the carbophenoxy group in II was readily determined by its distinctive "active ester" carbonyl at 1720 cm.$^{-1}$ in the in-



a. $R_1 = R_2 = C_2H_5$—
b. $R_1 = H$; $R_2 = n\text{-}C_4H_9$—

frared. The urea III, of course, did not show carbonyl absorption at higher frequency than that of the carboxyl at 1640 cm.$^{-1}$ Mixtures of II and III obtained in the earlier runs were readily separated by the high solubility of II, but relative insolubility of the urea III, in ethanol.

All other compounds could be prepared by this method, except 4-(carbophenoxyglycylamino)salicylic acid. In the latter case, sodium bicarbonate was not strong enough to remove the proton from the zwitterion structure of 4-(glycylamino)salicylic acid and it was necessary to use aqueous sodium hydroxide. If too much base was employed, the carbophenoxy group suffered cleavage, thus leading to unchanged amino acid; in addition, a shortened contact time with the aqueous base was advantageous.

To study carbophenoxy compounds such as II as potential irreversible inhibitors of LDH or GDH it was necessary to determine the stability of II in Tris buffer at pH 7.4 and 37°. Two possible reactions could take place, leading to one of three products. First, the phenoxy group might be displaced by the tris-(hydroxymethyl)aminomethane to give a mixed urea of type VI. Second, hydrolysis to *p*-aminosalicylic acid (I) might occur; the latter then might react with unchanged II to give the symmetrical urea III. To follow the course of this decomposition reaction, a model compound (VI*b*) was prepared in excellent yield by reaction of the carbophenoxy salicylic acid (II) with excess *n*-butylamine in dimethylformamide by short heating on a steam bath.

Table I lists the ultraviolet absorption spectra of the compounds involved in the decomposition of the carbophenoxy group of II. Although there was little difference in the position of the two peaks, the ϵ ratio of these two peaks was considerably higher in compound II than in the others; thus by following the change in the ϵ ratios it was possible to check the stability of II.

TABLE I.—RELATIVE ULTRAVIOLET PEAK RATIOS OF SUBSTITUTED 4-AMINOSALICYLIC ACIDS IN TRIS BUFFER (pH 7.4)

Compd.	Peak I, mμ (ϵ)	Peak II, mμ (ϵ)	ϵ Ratio of Peak I to Peak II
I	267 (13,000)	300 (8600)	1.51
II	264 (20,000)	300 (7750)	2.60
VI*b*	268 (20,800)	300 (10,000)	2.08

In 1% aqueous sodium bicarbonate (pH 8.4) at 37°, the ϵ ratio rapidly dropped from 2.60 to 1.75 in 15 minutes and 1.57 in 45 minutes; after 24 hours at 37°, the ϵ ratio was 1.50, the same as *p*-aminosalicylic acid (I). It is highly probable that at the high dilution this experiment is run (0.55 m*M*), hydrolysis to I occurs rather than urea III formation.

In Tris buffer (pH 7.4) at 37°, II was considerably more stable. In 1 hour, the ϵ ratio dropped from 2.60 to 2.42; after 24 hours at 37°, the ϵ ratio was 2.01. The latter number is probably within experimental error for formation of a mixed urea such as VI with the Tris; it is unlikely that hydrolysis to I (ratio of 1.51) took place since a greater change in ϵ ratio from 1 hour to 24 hours should have taken place. In either case, it is clear that the maximum decomposition that could occur in 1 hour in the Tris buffer (the length of the enzyme incubation period) was about 34% if the mixed urea VI with Tris was formed and less if I was formed.

The ease with which the phenoxy (II) could be converted to a mixed N-*n*-butyl-urea (VI*b*) with *n*-butylamine suggested that the synthesis of the mixed urea V with aziridine be attempted, since V could be a potentially useful irreversible inhibitor. When II was reacted with 3–4 equivalents of aziridine in dimethylformamide at about 90°, these conditions employed for preparation of VI*b* led to polymeric materials. That diethylamine could react smoothly with II to give a mixed urea (VI*a*) showed that a secondary amine could be used for this reaction. When a solution of II in dimethylformamide containing two equivalents of triethylamine was allowed to react with one equivalent of aziridine at room temperature, then the reaction mixture poured into dilute hydrochloric acid, slow separation of the chloroethyl urea IV took place over several days. That IV could be isolated certainly indicated the aziridinyl urea V had been formed. However, attempts to isolate V directly when the reaction was run or worked up under a variety of conditions proved to be fruitless, even though mixed ureas of aziridine have been previously prepared by the carbophenoxy route (13).

4-(Carbophenoxyamino)salicylic Acid (II).—A solution of 5.0 Gm. (32.6 mmoles) of 4-aminosalicylic acid in 130 ml. of water containing 11 Gm. of sodium bicarbonate was cooled to 3° in an ice bath. Then 7.55 Gm. (48 mmoles) of phenyl chloroformate

was added in one portion; the mixture was vigorously stirred with a mechanical paddle stirrer for 3 hours. During this time, the temperature stayed between 3–8°; some of the sodium salt of the product separated from solution. The solid sodium salt was collected on a filter and the filtrate was acidified to about pH 2 with 3 *N* hydrochloric acid. The product was collected on a filter, washed with water, and dried.

The insoluble sodium salt was quickly dissolved in 200 ml. of water preheated to about 75°, then filtered. The filtrate was immediately acidified with 3 *N* hydrochloric acid. The separated product was combined with the first fraction and recrystallized from aqueous ethanol; yield, 7.9 Gm. (89%) of pure material, m.p. 215–217°; $\nu_{max.}^{KBr}$ 3340 (NH); 1720 (urethane C=O); 1637 cm.$^{-1}$ (carboxyl C=O); $\lambda_{max.}^{EtOH}$ 264 (ϵ 25,000), 302 mμ (ϵ 9,800).

Anal.—Calcd. for $C_{14}H_{11}NO_5$: C, 61.5; H, 4.02; N, 5.12. Found: C, 61.3; H, 4.06; N, 5.26.

5-(Carbophenoxyamino)salicylic Acid.—This compound was prepared from 5-aminosalicylic acid hydrochloride in the same manner as described for II. Recrystallization from alcohol-water gave 7.0 Gm. (97%) of product as nearly white crystals, m.p. 264–275° dec.; $\lambda_{max.}^{EtOH}$ 221 (ϵ 41,200), 246 (ϵ 16,900), 325 mμ (ϵ 3,800); $\nu_{max.}^{KBr}$ 3310(NH); 1705 (urethane C=O); 1620 cm.$^{-1}$ (carboxyl C=O).

Anal.—Calcd. for $C_{14}H_{11}NO_5$: C, 61.5; H, 4.02; N, 5.12. Found: C, 61.3; H, 3.78; N, 5.31.

m-(Carbophenoxyamino)oxanilic Acid.—From 5 Gm. of *m*-aminooxanilic acid (14) and 5.9 Gm. of phenyl chloroformate, as described for the preparation of II, a 4.5-Gm. (59%) quantity of product was obtained. Recrystallization from chloroform afforded white crystals, m.p. 182–183°; $\lambda_{max.}^{EtOH}$ 237 mμ (ϵ 35,900); $\nu_{max.}^{KBr}$ 3310 (NH); 1760 (urethane C=O); 1710 (carboxyl C=O); 1680 cm.$^{-1}$ (amide C=O).

Anal.—Calcd. for $C_{15}H_{12}N_2O_5$: C, 60.0; H, 4.00; N, 9.33. Found: C, 59.9; H, 4.16; N, 9.29.

4-(Carbophenoxyglycylamino)salicylic Acid.—A solution of 1.00 Gm. (4.76 mmoles) of 4-(glycylamino)salicylic acid (12, 15) in 40 ml. of 0.25 *N* sodium hydroxide was cooled in an ice bath. When the temperature reached 3°, 1.14 Gm. (7.14 mmoles) of phenyl chloroformate was added in one portion. The mixture was rapidly mixed in with a mechanical paddle stirrer with continued ice bath cooling for 90 minutes. The cold solution was filtered through a Celite pad and the filtrate immediately acidified with 3 *N* hydrochloric acid. The product was collected on a filter and washed with water; yield, 0.70 Gm. (45%), m.p. 236–238°. Recrystallization from ethyl acetate gave white crystals, m.p. 242–243°; $\nu_{max.}^{KBr}$ 3400 (NH); 1720 (sh.) (urethane C=O); 1640 (carboxyl C=O); 1680 (amide C=O); 732, 690 cm.$^{-1}$ (monosubstituted phenyl). Longer reaction times or more base gave lower yields.

Anal.—Calcd. for $C_{16}H_{14}N_2O_6$: C, 58.2; H, 4.24; N, 8.48. Found: C, 58.1; H, 4.44; N, 8.29.

N - n - Butyl - N′ - (3 - hydroxy - 4 - carboxyphenyl)urea (VI*b*).—To a solution of 1.00 Gm. (3.66 mmoles) of 4-(carbophenoxyamino)salicylic acid (II) in 10 ml. of dimethylformamide was added 0.80 Gm. (11 mmoles) of *n*-butylamine. After being heated on a steam bath for 1 hour protected from moisture, the solution was diluted with 40 ml. of water and acidified to about pH 3 with 3 *N* hydrochloric acid. The product was collected on a filter and washed with water. Recrystallization from dilute alcohol gave 0.80 Gm. (95%) of white crystals, m.p. 205–206°; $\nu_{max.}^{KBr}$ 3325 (NH), 1650 (urea and carboxyl C=O); and no urethane C=O near 1720 cm.$^{-1}$; $\lambda_{max.}^{EtOH}$ 274 (19,800), 302 mμ (ϵ 12,300).

Anal.—Calcd. for $C_{12}H_{16}N_2O_4$: C, 57.1; H, 6.34; N, 11.1. Found: C, 57.5; H, 6.57; N, 11.3.

N,N - Diethyl - N′ - (3 - hydroxy - 4 - carboxyphenyl)urea (VI*a*).—This was prepared in the same manner as described for VI*b* using diethylamine; yield, 0.90 Gm. (98%). Recrystallization from dilute alcohol gave white crystals, m.p. 161–162°; $\nu_{max.}^{KBr}$ 3450 (NH); 1640 (urea and carboxyl C=O); and no urethane C=O near 1720 cm.$^{-1}$; $\lambda_{max.}^{EtOH}$ 273 (ϵ 19,400), 304 mμ (ϵ 23,500).

Anal.—Calcd. for $C_{12}H_{16}N_2O_4$: C, 57.1; H, 6.34; N, 11.1. Found: C, 57.1; H, 6.35; N, 10.8.

N - (2 - Chloroethyl) - N′ - (3 - hydroxy - 4 - carboxyphenyl)urea (IV).—To a solution of 1.00 Gm. (3.66 mmoles) of 4-(carbophenoxyamino)salicylic acid (II) and 1.02 ml. (7.32 mmoles) of triethylamine in 10 ml. of dimethylformamide a 0.188-ml.

TABLE II.—IRREVERSIBLE INHIBITION OF LDH AND GDH BY CARBOPHENOXY COMPOUNDS

	GDH						LDH					
		Inactivation, Rate		Saturation Rate				Inactivation, Rate		Saturation Rate		
Compd.	I_{50}	mM Concn.	Rate[a]	mM Concn.	Ratio[b] Calcd.	Found	I_{50}	mM Concn.	Rate[a]	mM Concn.	Ratio[b] Calcd.	Found
4-(Iodoacetamido)-salicylic acid[c]	3.4	2	1.0	2/1	1.26	1.23	6.6	2	1.0	3/1	1.93	2.0
4-(Carbophenoxy-amino)salicylic acid (II)	3.4	1.5	0, 0				4.4	2	0.98	2/1	1.34	2.6, 3.7
		3.0	0, 0	—	—	—		2	0.85	4/2	1.23	2.3
		2	0, 0, 0							4/2		3.4[d]
5-(Carbophenoxy-amino)salicylic acid (VII)	1.6	2	2.0	2/1	1.17	1.1	5.9	2	0, 0			
		2	2.0	2/1		1.2, 1.0		2	0, 0	—	—	—
								2	0.1, 0.1			
4-(Carbophenoxy-glycylamino)sali-cylic acid (VIII)	6.7	2	1.3	4/2	1.20	1.0	6.3	2	0[e]			
		2	1.6	4/2		1.2		2	0	—	—	—
m-(Carbophenoxy-amino)oxanilic acid (IX)	4.0	2	1.85	2/1	1.21	1.3	0.87	2	0[e]			
		2	1.87	2/1		1.3		2	0[e]	—	—	—
								2	0[e]			

[a] Rate compared to 4-(iodoacetamido)salicylic acid, the standard that is assigned an arbitrary value of 1.0 and is run simultaneously. [b] Ratio of rates of inactivation when run simultaneously. [c] The figures on 4-(iodoacetamido)salicylic acid were previously reported in Reference 6. [d] Different preparation of inhibitor than those preceding. [e] This value was less than zero; that is, the compound protected the enzyme against the small amount of thermal inactivation observed in the control.

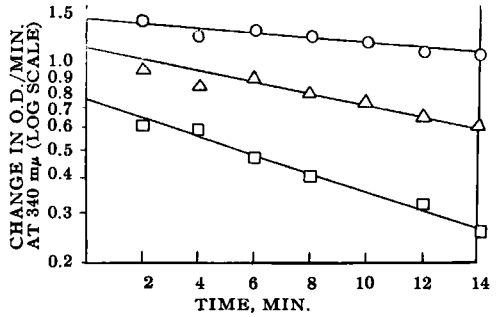


Fig. 1.—Comparative irreversible inhibition of GDH·DPNH by 4-ISA and 5-(carbophenoxyamino) salicylic acid (VII). Key: O, GDH·DPNH control; □, 2 m*M* 5-(carbophenoxyamino)salicylic acid; △, 2 m*M* 4-ISA.

(3.66 mmoles) quantity of aziridine was added. After standing about 18 hours in a stoppered flask, the solution was diluted with 40 ml. of water and strongly acidified with 3 *N* hydrochloric acid. During a period of 1 week at about 3°, white crystals gradually separated; yield, 0.3 Gm. (37%), m.p. 177–179°. Recrystallization from absolute alcohol–petroleum ether gave white crystals, m.p. 185–187°; $\nu_{max.}^{KBr}$ 3375 (NH); 1650 (urea and carboxyl C=O); and no urethane near 1720 cm.$^{-1}$ The compound gave a positive test for halogen.

Anal.—Calcd. for $C_{10}H_{11}ClN_2O_4$: C, 46.5; H, 4.26; N, 10.9. Found: C, 46.5; H, 4.46; N, 10.9.

N - (2 - Chloroethyl) - N′ - (3 - carboxy - 4 - hydroxyphenyl)urea.—This was prepared from 5-(carbophenoxyamino)salicylic acid in 50% yield, m.p.

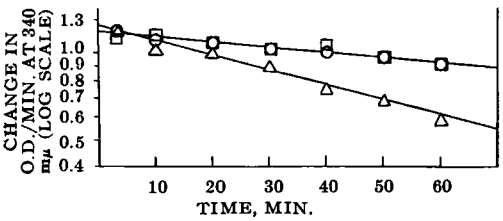


Fig. 2.—Comparative irreversible inhibition of LDH·DPNH by 4-ISA and 5-(carbophenoxyamino)-salicylic acid (VII). Key: O, LDH·DPNH control; □, 2 m*M* 5-(carbophenoxyamino)salicylic acid; △, 2 m*M* 4-ISA.

180–183°. Recrystallization from absolute alcohol–petroleum ether gave white crystals, m.p. 188–189°.

Anal.—Calcd. for $C_{10}H_{11}ClN_2O_4$: C, 46.5; H, 4.26; N, 10.9. Found: C, 46.9; H, 4.13; N, 11.1.

RESULTS

Comparisons of the reversible and irreversible inhibition of LDH and GDH by the four carbophenoxy derivatives are listed in Table II. All four compounds were good to excellent reversible inhibitors of both enzymes. Three of the four compounds, 5-(carbophenoxyamino)salicylic acid (VII), 4-(carbophenoxyglycylamino)salicylic acid (VIII), and *m*-(carbophenoxyamino)oxanilic acid (IX) inactivated GDH even more rapidly than the standard compound, 4-(iodoacetamido)salicylic acid; in contrast, none of these three compounds showed inactivation of LDH. These three compounds also showed a rate saturation in inactivation of GDH.

The theoretical rate saturations values can be calculated in the following manner if the rate of inactivation is dependent upon the concentration of reversible complex, *EI*, formed in Eq. 1.

$$E + I \longleftrightarrow EI \qquad \text{(Eq. 1)}$$

where E is the free enzyme concentration, I is the inhibitor concentration and EI is the concentration of enzyme-inhibitor reversible complex.

$$K_I = \frac{[E]\,[I]}{[EI]} \qquad \text{(Eq. 2)}$$

$$E_t = [E] + [EI] \qquad \text{(Eq. 3)}$$

where E_t = the total enzyme concentration excluding that portion of the enzyme that has become inactivated.

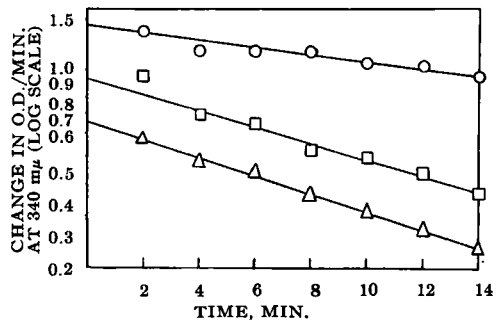


Fig. 3.—Rate saturation effect during inactivation of GDH·DPNH by 5-(carbophenoxyamino)salicylic acid (VII). Key: O, GDH·DPNH control; □, 1 m*M* VII; △, 2 m*M* VII.

Substituting Eq. 3 in Eq. 2 gives Eq. 4

$$[EI] = \frac{[E_t]}{\frac{K_I}{[I]} + 1} \qquad \text{(Eq. 4)}$$

Similarly, Eq. 5 can be derived (16) where S = the substrate concentration and ES = the concentration of enzyme-substrate complex and Km = the substrate-enzyme dissociation constant

$$[ES] = \frac{E_t}{\frac{Km}{[S]} + 1} \qquad \text{(Eq. 5)}$$

When $[ES] = [EI]$, 50% inhibition will be obtained, the I_{50}. Thus, equating Eqs. 4 and 5 gives Eq. 6

$$K_I = \frac{Km[I]}{[S]} = Km \times I_{50} \qquad \text{(Eq. 6)}$$

Since $Km = 2.5 \times 10^{-4}$ for α-oxoglutarate on GDH has previously been determined (6), the K_I for any of the compounds in Table II can be calculated. Thus, from Eq. 6 and an I_{50} of 1.6, 5-(carbophenoxyamino)salicylic acid (VII) has $K_I = 4 \times 10^{-4}$. Then when $[I] = 2$, Eq. 4 gives $[EI] = 0.83\,[E_t]$ and when $[I] = 1$, $EI = 0.71\,[E_t]$. An increase in $[I]$ from 1 m*M* to 2 m*M* should increase $[EI]$ by 0.83/0.71 or 1.17-fold. Therefore, the ratio of rates of inactivation should be 1.17 if the inactivation is

dependent upon initial reversible complex at the active site (exo-alkylation) and should be 2.0 if the inactivation were due to a random bimolecular collision between inhibitor and enzyme (tail-alkylation).

Other rate saturation ratios calculated similarly are listed in Table II. In the case of compound VIII, where the I_{50} value is greater than five it is better to use a 4/2 ratio to show rate saturation, rather than the standard 2/1 ratio. It is clear that compounds VII, VIII, and IX (Table II) inactivate GDH with rate saturation in agreement with the prediction for the exo-alkylation mechanism.

Not all carbophenoxy compounds showed inactivation of GDH, as the bridge principle would predict. Thus, 4-(carbophenoxyamino)salicylic acid (II) showed no irreversible inhibition of GDH in four runs. Irreversible inhibition of LDH at a slightly slower rate than the standard 4-ISA was shown by II; since II did not show a rate saturation effect, this irreversible inactivation of LDH by II is by some mechanism different than the exo-alkylation mechanism. (See *Discussion*.)

To conserve space, only three of the many inactivation runs listed in Table II are shown. Figure 1 shows the inactivation of GDH by 5-(carbophenoxyamino)salicylic acid (VII) and Fig. 2 shows the lack of inactivation of LDH by VII. A rate saturation effect in the inactivation of GDH by VII is shown in Fig. 3.

DISCUSSION

In seeking evidence for the exo-alkylation phenomenon (3), we deliberately chose the experimental design of an alkylating group with low specificity; in this way, the chances were increased that the alkylating group could covalently link with some enzymic nucleophilic site adjacent to the active site after a reversible complex had been formed at the active site. A group with the desired low specificity was the iodoacetyl group; strong experimental evidence was obtained (5, 6) that 4-(iodoacetamido)-salicylic acid inactivated both LDH and GDH by the exo-alkylation mechanism. It was then proposed (6) that the ability of the alkylating group to bind covalently with an enzymic nucleophilic site was dependent both on the ability of the bound inhibitor to bridge to the nucleophilic group and upon the nucleophilicity of the enzymic site being alkylated. Such an hypothesis, if true, could give an extra magnitude of specificity not possible with reversible inhibitors.

Specificity with the iodoacetyl group was obtained by influencing the bridging (9, 10), even though the iodoacetyl group has little functional specificity. Of some 15 amino acids in proteins containing a third functional group, about one-half have the nucleophilic capacity to react with the iodoacetyl group to form a covalent bond. In contrast, the carbophenoxy group of a molecule such as 5-(carbophenoxyamino)salicylic acid (VII), has complete functional specificity; that is, it will react only with a primary amino group on a protein; neither the indole-NH of tryptophane or the imidazole-NH of histidine will react with phenyl esters (17). Although no information concerning the reaction of phenyl esters with the guanidino group of arginine could be found, the guanidino group is not a probable site since it would be fully protonated at pH 7.4. Thus, the most likely nucleophilic site of a protein that could bind covalently with the carbophenoxy group by exo-alkylation would be the ϵ-amino group of a lysine and, less likely, a terminal α-amino group.[2]

All four carbophenoxy compounds listed in Table II were good to excellent reversible inhibitors of LDH and GDH. Of these, three (VII–IX) irreversibly inactivated GDH but had no inactivation effect on LDH; it is highly probable that these three compounds inactivated GDH by the exo-alkylation mechanism since all three showed the rate saturation effect indicating that a reversible complex between GDH and the inhibitor was an obligatory intermediate to inactivation. Although it would appear that the inactivity of II is because of the inability of the urethane group of 4-(carbophenoxyamino)salicylic acid (II) to reach the enzymic amino group for covalent bond formation, it is also possible that the enzyme hinders the relatively large phenyl group from assuming the transition state necessary for covalent bond formation.

The fact that GDH is attacked by either the carbophenoxy group of VII–IX or by the iodoacetyl group of 4- or 5-(iodoacetamido)salicylic acid (6) indicates that within the reversible enzyme-inhibitor complex an amino group on the enzyme can be bridged for covalent bond formation; however, it cannot necessarily be concluded that the amino group on GDH covalently bound by VII–IX is the same group that is covalently bound by the (iodoacetamido)salicylic acids, even though it is possible for these two nucleophilic groups to be one and the same.

Conversely, it can be concluded that VII, VIII, and IX cannot bridge to an available amino group on LDH. It might have been expected that 4-(carbophenoxyglycylamino)salicylic acid (VIII), with its relatively long bridging ability, should have been able to form a covalent bond with an amino group on LDH; however, it is also possible that hindrance interaction between the enzyme and the phenyl group prevented the formation of the proper transition state. If a carbophenoxy compound is ultimately found that can irreversibly inhibit two enzymes, it would be of interest to see if specificity could be achieved by placing bulky groups on the phenoxy radical for selective hindrance of the transitional state.

Although 4-(carbophenoxyamino)salicylic acid (II) inactivated LDH almost as rapidly as the standard, 4-ISA, it is clear that this inactivation did not proceed by the exo-alkylation mechanism since no rate saturation effect was obtained. Several obvious explanations are readily eliminated: (*a*) the inactivation is not by the tail-alkylation mechanism, or VII, VIII, and IX should also have inactivated LDH by this bimolecular process; (*b*) it is highly unlikely that some extraneous impurity caused this effect, since two different samples prepared at 6-month intervals showed the same effect.

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[2] As pointed out by a referee, the carbophenoxy group is an acylating group and not an alkylating group. At the time that the first evidence for the exo-alkylation phenomenon was presented (5), an acylation reaction of this type was unforeseen. We have used "exo-alkylation" as equivalent to "covalent bond formation—adjacent to the binding site—within the enzyme-inhibitor reversible complex." It is apparent that the latter general expression was intended and that perhaps the word "exo-alkylation" should be literally limited to covalent bond formation by alkylation.

A possible explanation is that 4-(carbophenoxyamino)salicylic acid (II) can bind weakly to some site other than the active site, then form a covalent link that makes the active site inaccessible to the substrate; such reversible binding to an extraneous site would have to have a dissociation constant larger than 10^{-2} to show the lack of a rate saturation effect within experimental error. The fact that one compound does not show a rate saturation effect actually strengthens the interpretation that those compounds showing a rate saturation effect might operate by the exo-alkylation mechanism.

Although no compound in Table II inactivated LDH selectively by the exo-alkylation mechanism, it is probable that such a compound could be found by further investigation.

Of importance to chemotherapy is the irreversible specificity noted with the phenyl esters (VII–IX), this specificity being because of the difference in the nucleophilic character of the enzymic groups being covalently bound on LDH and GDH. Groups on a reversible inhibitor that can bridge to and specifically bind other enzymic functional groups would be of use in both chemotherapy and protein structure studies; such a study is continuing in these laboratories.

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Nonclassical Antimetabolites XIII

Simulation of the 5′-Phosphoribosyl Moiety of 5′-Adenylic Acid at the Enzyme Level by ω-Carboxyalkyl and Aralkyl Groups Attached to Adenine

By B. R. BAKER and H. S. SACHDEV

5′-Adenylic acid can inhibit both lactic dehydrogenase and glutamic dehydrogenase; since both the phosphate and adenine moieties are necessary for good inhibition, the 5′-adenylic acid presumably competes with DPN. This inhibition by 5′-adenylic acid can be duplicated by 9-(4′-carboxybutyl)-adenine (XVIII) on both enzymes, indicating that the ω-valeric acid side chain can simulate the binding of the 5-phosphoribosyl group when the latter is attached to adenine. 9-(*para*-Carboxybenzyl)-adenine (XVI), a compound with a conformation-fixed side chain, can simulate the binding of 5′-adenylic acid to glutamic dehydrogenase but not lactic dehydrogenase; this difference has been attributed to the difference in conformation of 5′-adenylic acid (and DPN) when bound to the two enzymes.

MANY IMPORTANT ENZYMES have substrates bearing the 5-phosphoribosyl moiety such as the ribotides involved in nucleic acid biosynthesis. A number of purines and pyrimidines useful in cancer chemotherapy operate by enzymic blockade at the nucleotide level (2). For example, 5-fluorouracil is converted intracellularly to 5-fluorouracil deoxyribotide; the latter inhibits cell growth by blockade of thymidylate synthetase (3, 4). Similarly, in the cell 6-mercaptopurine (I) is converted by inosinic pyrophosphorylase to 6-mercaptopurine ribotide (II) (5, 6). The latter compound is a potent inhibitor for inosinic dehydrogenase, the enzyme converting inosinic acid (IV) to xanthylic acid (V); at a somewhat higher concentration, II also inhibits adenylosuccinate synthetase, the enzyme forming adenylosuccinate (III) from inosinic acid (IV) (7, 8).

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Of importance to chemotherapy is the irreversible specificity noted with the phenyl esters (VII–IX), this specificity being because of the difference in the nucleophilic character of the enzymic groups being covalently bound on LDH and GDH. Groups on a reversible inhibitor that can bridge to and specifically bind other enzymic functional groups would be of use in both chemotherapy and protein structure studies; such a study is continuing in these laboratories.

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5′-Adenylic acid can inhibit both lactic dehydrogenase and glutamic dehydrogenase; since both the phosphate and adenine moieties are necessary for good inhibition, the 5′-adenylic acid presumably competes with DPN. This inhibition by 5′-adenylic acid can be duplicated by 9-(4′-carboxybutyl)-adenine (XVIII) on both enzymes, indicating that the ω-valeric acid side chain can simulate the binding of the 5-phosphoribosyl group when the latter is attached to adenine. 9-(*para*-Carboxybenzyl)-adenine (XVI), a compound with a conformation-fixed side chain, can simulate the binding of 5′-adenylic acid to glutamic dehydrogenase but not lactic dehydrogenase; this difference has been attributed to the difference in conformation of 5′-adenylic acid (and DPN) when bound to the two enzymes.

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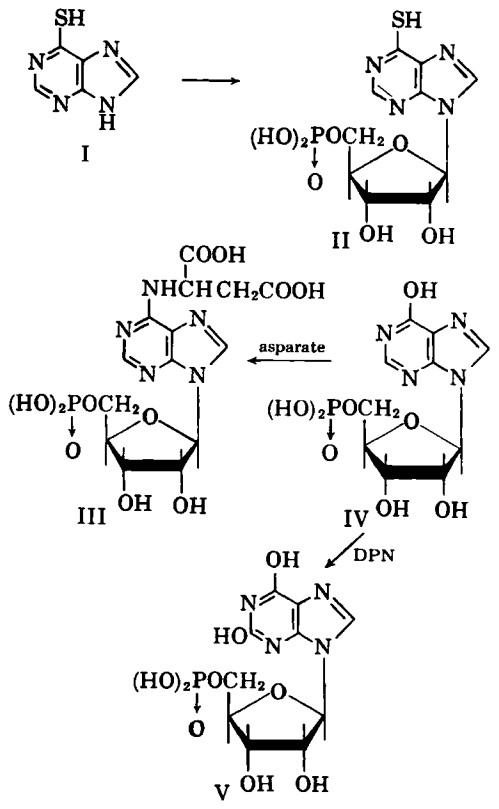
Unfortunately, cancer cells all too soon become resistant to the effects of 6-mercaptopurine (I) because of the mutational loss of the inosinic pyrophosphorylase that converts I and hypoxanthine to their ribonucleotides, II and IV (5, 8). Although 6-mercaptopurine ribotide (II) is still effective as an inhibitor of the enzymes utilizing inosinic acid (IV) in a cell-free extract of resistant cell lines (7), II has little effect on the intact resistant cells (7, 9, 10). This lack of activity probably is because 6-mercaptopurine ribonucleotide (II), like natural nucleotides (11), suffers enzymic cleavage back to the corresponding base (I) during entrance into the cell by active transport.

One approach to the solution of the enigma of this resistance problem has been to block completely the phosphate group of II as a triester (12); the latter type could presumably enter the cell by passive transport, then be reconverted intracellularly to the free nucleotide (II) by a nonspecific phosphoesterase. This approach has been partially successful (13).

A second approach would be to utilize a 9-substituted derivative of 6-mercaptopurine wherein this 9-substituent could simulate the binding of the ribotide moiety of II to the enzymes utilizing inosinic acid (IV) as a substrate but still have the ability to penetrate a cell wall and not suffer enzymic cleavage to the base. Since a major portion of the binding of the ribose phosphate moiety can certainly be attributed to the binding of the ionized phosphate to an enzymic cationic site, other anionic groups such as sulfates, phosphonates, or carboxylates should be able to replace the phosphate group in binding. In addition, since the ribose part of this moiety probably contributes much less than half of the total binding of this moiety, the ribose fragment should be replaceable by any group that is smaller than β-D-ribofuranose, providing that this group can properly juxtaposition the anionic binding group and the purine moiety. This juxtapositioning will be dependent upon the conformation of the nucleotide as it binds to a given enzyme. For reasons to be presented in the *Discussion*, the *p*-carboxybenzyl and the 4-carboxybutyl groups attached to the 9-position of some purines were selected for initial investigation.



Although crude preparations of inosinic dehydrogenase (14, 15) and adenylosuccinate synthetase (16) (two enzymes utilizing inosinic acid (IV)) have been described, we considered it expedient to use commercially available crystalline enzymes to determine—as a first approximation—if the binding of a nucleotide to one of these enzymes could be simulated by replacement of the ribose phosphate moiety with a suitable group. Since LDH and GDH[1] have been used extensively in these laboratories for other inhibitor studies (1, 17, 18), and since both enzymes use the dinucleotide, DPN, as a cofactor, these two enzymes were selected for initial studies of the relative binding of AMP, 9-(*p*-carboxybenzyl)adenine (XVI), and 9-(4′-carboxybutyl)-hypoxanthine (XVIII). The synthesis and evaluation of the latter two compounds as DPN inhibitors of LDH and GDH is the subject of this paper.

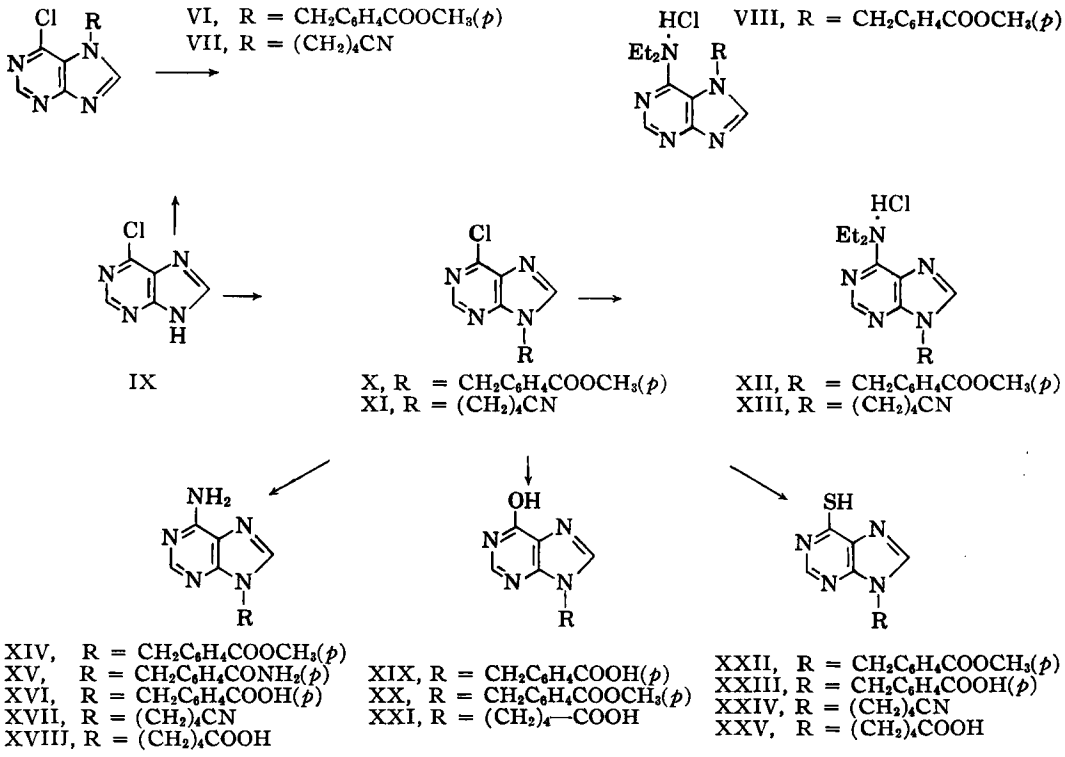
EXPERIMENTAL

Enzyme Measurements

Reagents.—DL-Lactate, L-glutamate, DPN, and adenosine were purchased from Nutritional Biochemicals Corp. AMP, IMP, and AMP-2′(3′) were purchased from Pabst Laboratories. Crystalline LDH (isolated from rabbit skeletal muscle) and crystalline GDH (isolated from mammalian liver) were purchased from the Sigma Chemical Co.

Methods of Assay.—The enzymatic rates for LDH and GDH were determined by the rate of formation of DPNH as measured by the rate of

[1] Abbreviations used: LDH, lactic dehydrogenase; GDH, glutamic dehydrogenase; DPN, diphosphopyridine nucleotide; AMP, 5′-adenylic acid; IMP, 5′-inosinic acid; AMP-2′(3′), a 1:1 mixture of 2′- and 3′-adenylic acid; Ad, adenine; Hx, hypoxanthine; ADP, adenosine 5′-diphosphate.



appearance of DPNH at 340 mμ in a Cary 11 spectrophotometer. All reactions were run in 0.05 *M* Tris buffer (pH 8.4).

For LDH, the cell contained 3.2 m*M* DPN, 8 m*M* DL-lactate, and the inhibitor when necessary in a total volume of 3.0 ml. The reaction was initiated by the addition of the appropriate amount of enzyme in 100 λ of buffer. Sufficient enzyme was used to give an initial rate of formation of DPNH of 0.4–0.6 optical density units per minute (17, 19).

The GDH assays were run similarly using 3.2 m*M* DPN, 2 m*M* L-glutamate and the inhibitor when necessary. Sufficient enzyme was used to give an initial rate of 0.9–1.2 optical density units per minute (17, 20).

Chemistry

Methods.—Although some purines can be alkylated directly to 9-alkylpurines–for example, 2-methylthio-6-dimethylaminopurine (21) and purine (22)–other purines react poorly or even fail to react properly (23). More often mixtures of 7- and 9-alkyl derivatives are obtained which must be separated (21, 24). The elegant methods developed by Montgomery and Temple (24, 25) for synthesis of 9-alkylpurines *via* 9-alkyl-6-chloropurine are particularly suitable if one wishes to have several different substituents at the 6-position. Their first route for 9-alkyl-6-chloropurines *via* 5-amino-4,6-dichloropyrimidine and an appropriate amine is favored if the necessary alkylamine is readily available, since only a 9-substituted purine can be obtained after ring closure with ethyl orthoformate-acetic anhydride (25–28). The second route involving the reaction of 6-chloropurine with an alkyl halide is useful when an alkyl halide is more available than the amine; dimethylformamide or dimethylsulfoxide as the solvent and potassium carbonate as the acid acceptor were the keys to the success of this reaction (24, 29). In most cases a mixture of 7- and 9-alkyl-6-chloropurines were obtained with the 9-isomer predominating as is usually the case in such alkylations (21). Once the separation has been completed, the chloro group can then be displaced by a large variety of nucleophiles (24, 25, 29) in a direct manner that gives no further isomer problems.

The second route of Montgomery and Temple was chosen for our syntheses of carboxyalkyl and aralkyl purines since bromo esters give considerably less difficulty in nucleophilic reactions than amino esters; the latter cause complications by polymerization. Condensation of methyl α-bromo-*p*-toluate (30) with 6-chloropurine (IX) proceeded smoothly. The 9-substituted purine (X) was obtained in 65% yield and was readily separated from the 7-isomer (VI) formed in 4% yield; the latter is readily soluble in hot methanol and X is insoluble. That the major isomer was the 9-isomer (X) was readily shown by conversion of X and VI to the corresponding 6-diethylaminopurines (XII and VIII) by the use of diethylamine in boiling methanol. The two 6-diethylaminopurines were readily distinguished by the large difference in their ultraviolet absorption maxima; XII showed a peak at 276 mμ in ethanol, whereas VIII showed a maximum at 298 mμ, in agreement with other 7- and 9-alkyl-6-dimethylaminopurines (21). The use of diethylamine is simpler than the use of dimethylamine for these structure proofs (24), since dimethylamine requires a sealed vessel for reaction.

Reaction of 9-(*p*-carbomethoxybenzyl)-6-chloropurine (X) with methanolic ammonia at 105° for 75 minutes gave the corresponding adenine derivative (XIV) in 85% yield. However, longer reaction

times and higher temperatures (115°) or both led to the additional ammonolysis of the ester function with formation of appreciable quantities of the amide (XV). Saponification of XIV with hot 1 *N* sodium hydroxide afforded the desired 9-(*p*-carboxybenzyl)-adenine (XVI) in 80% yield.

When the chloropurine, X, was refluxed with concentrated hydrochloric acid for 2 hours, hydrolysis of the 6-chloro and ester groups occurred with formation of 9-(*p*-carboxybenzyl)-hypoxanthine (XIX) in 89% yield. The use of hot 0.1 *N* hydrochloric acid gave only hydrolysis of the 6-chloro group; the resultant hypoxanthine ester (XX) was isolated in 74% yield.

The chloropurine X also reacted smoothly with thiourea in boiling methanol to give the 6-mercaptopurine ester XXII in 90% yield. Further saponification of XXII with hot 1 *N* sodium hydroxide afforded 9-(*p*-carboxybenzyl)-6-mercaptopurine (XXIII) in 70% yield.

Alkylation of 6-chloropurine (IX) with 5-bromovaleronitrile in dimethylformamide proceeded smoothly. Apparently considerable amounts of 7-isomer were present which made purification somewhat difficult and the yield of usable 9-isomer (XI) was 27%. That the crystalline isomer obtained was indeed the 9-isomer was shown by conversion of XI to the 6-diethylaminopurine derivative (XIII), isolated as the crystalline hydrochloride; the latter showed an ultraviolet maximum at 276 mμ in ethanol, as expected for a 9-isomer (21). Conversion of XI to 9-(4′-carboxybutyl)-hypoxanthine (XXI), 9-(4′-cyanobutyl)-6-mercaptopurine (XXIV) and 9-(4′-cyanobutyl)-adenine (XVII) proceeded smoothly as described for the *p*-carboxybenzyl series. However, conversion of XXIV and XVII to the corresponding 9-(4′-carboxybutyl)-6-mercaptopurine (XXV) and 9-(4′-carboxybutyl)-adenine (XVIII), respectively, was more expediently done with hot concentrated hydrochloric acid than with the alkaline hydrolysis used in the *p*-carboxybenzyl series.

Synthesis.[2]—*9-(p-Carbomethoxybenzyl)-6-chloropurine (X).*—To a solution of 6.87 Gm. (0.03 mole) of methyl α-bromo-*p*-toluate (30) in 50 ml. of dimethylformamide was added 4.4 Gm. (0.032 mole) of anhydrous potassium carbonate followed by 4.63 Gm. (0.03 mole) of 6-chloropurine. The mixture was stirred for 2 hours protected from moisture during which time a buff-colored solid separated. The mixture was poured into 150 ml. of ice water. The solid was collected on a filter and washed with 50 ml. of water. The dry filter cake weighed 8.2 Gm., m.p. 165–190°. The dry solid was heated to boiling with 125 ml. of methanol and filtered hot. The undissolved product melted at 196–201°; yield, 5.9 Gm. (65%). Recrystallization from benzene gave analytically pure material; yield 4.2 Gm. (46%), m.p. 200–201°; $\nu_{max.}^{KBr}$ 1715 (ester); 1600, 1560, 1510 cm.$^{-1}$ (C=C, C=N).

Anal.—Calcd. for $C_{14}H_{11}ClN_4O_2$: C, 55.5; H, 3.70; N, 18.5. Found: C, 55.6; H, 3.83; N, 18.5.

The hot methanol mother liquor was evaporated to residue *in vacuo*. Two recrystallizations from methanol gave 0.40 Gm. (4%) of 7-(*p*-carbomethoxybenzyl-6-chloropurine (VI), m.p. 174°.

Anal.—Calcd. for $C_{14}H_{11}ClN_4O_2$: C, 55.5; H, 3.70; N, 18.5. Found: C, 55.6; H, 3.62; N, 18.4.

9-(4′-Cyanobutyl)-6-chloropurine (XI).—A mixture of 6.18 Gm. (0.04 mole) of 6-chloropurine (IX), 7.24 Gm. (0.044 mole) of 5-bromovaleronitrile, 5.52 Gm. (0.04 mole) of anhydrous potassium carbonate, and 60 ml. of dimethylformamide was stirred at room temperature for 8 hours. After dilution with 180 ml. of ice cold water, the mixture was extracted with chloroform (4 × 50 ml.). The combined extracts were dried with anhydrous magnesium sulfate and the solvent was spin-evaporated *in vacuo;* the last traces with dimethylformamide were removed at oil pump pressure. The oily residue solidified after standing overnight. Two recrystallizations from benzene-petroleum ether gave 2.5 Gm. (27%) of product, m.p. 87–89°, that was suitable for further transformations. Repeated recrystallization afforded the analytical sample, m.p. 90–91°; $\nu_{max.}^{KBr}$ 2250 (C≡N); 1590, 1550, 1490 cm.$^{-1}$ (C=C, C=N).

Anal.—Calcd. for $C_{10}H_{10}ClN_5$: C, 51.0; H, 4.28; N, 29.7. Found: C, 51.1; H, 4.31; N, 29.5.

9-(p-Carbomethoxybenzyl)-6-diethylaminopurine Hydrochloride (XII).—A 160-mg. (2.2 mmoles) quantity of diethylamine was added to a solution of 302 mg. (1 mmole) of X in 25 ml. of reagent grade methanol. After being refluxed for 2 hours, the solution was spin-evaporated to a syrup *in vacuo*. The residual gum was triturated with 15 ml. of 1:1 reagent acetone-dry ether; the diethylamine hydrochloride was removed by filtration and washed with 1:1 acetone-ether. With cooling, dry hydrogen chloride was passed into the filtrate until precipitation was complete; the product rapidly separated. It was collected on a filter and washed with ether. The solid was dissolved in reagent acetone and dry ether was added to turbidity. On standing at 3°, the pure product separated as white crystals; yield, 170 mg. (45%), m.p. 183–184°; $\lambda_{max.}^{EtOH}$ 276 mμ (ε 19,500).

Anal.—Calcd. for $C_{18}H_{22}ClN_5O_2$: C, 57.5; H, 5.91; N, 18.6. Found: C, 57.7; H, 6.05; N, 18.9.

No attempt was made to obtain a second crop.

9-(4′-Cyanobutyl)-6-diethylaminopurine Hydrochloride (XIII).—This was prepared in a similar way and recrystallized from 1:1 ethyl acetate–acetone; yield of pure material 52%, m.p. 120–123°; $\lambda_{max.}^{EtOH}$ 277 mμ (ε 20,000).

Anal.—Calcd. for $C_{14}H_{21}ClN_6$: C, 54.5; H, 6.86; N, 27.3. Found: C, 54.3; H, 6.96; N, 27.2.

7-(p-Carbomethoxybenzyl)-6-diethylaminopurine Hydrochloride (VIII).—This was also prepared as described for XII. Recrystallization from acetone–ether gave a 64% yield of pure product, m.p. 193–195°; $\lambda_{max.}^{EtOH}$ 298 mμ (ε 15,900).

Anal.—Calcd. for $C_{18}H_{22}ClN_5O_2$: C, 57.5; H, 5.91; N, 18.6. Found: C, 57.4; H, 6.01; N, 18.4.

9-(p-Carbomethoxybenzyl)-adenine (XIV).—A 1.00-Gm. (3.3 mmoles) quantity of X was added to 75 ml. of methanol saturated with ammonia gas at 0° and placed in a steel bomb. The bomb was heated at 105–106° for 75 minutes, then cooled in cold water. A white solid separated out on the walls of

[2] Melting points were taken on a Mel-temp. apparatus in capillary tubes and are uncorrected. Infrared spectra were determined in KBr disk with a Perkin-Elmer recording spectrophotometer 137B. Ultraviolet spectra were determined with a Cary 11 recording spectrophotometer.

the bomb. The product was collected on a filter and washed with a little cold methanol; yield, 0.80 Gm. (85%), m.p. 228–229°, that was sufficiently pure for further transformations. An analytical sample was obtained by recrystallization from methanol as white crystals, m.p. 230–231°; $\nu_{max.}^{KBr}$ 3300 (NH); 1730 (ester C=O); 1675, 1610, 1570 cm.$^{-1}$ (NH, C=C, C=N).

Anal.—Calcd. for $C_{14}H_{13}N_5O_2$: C, 59.4; H, 4.62; N, 24.7. Found: C, 59.2; H, 4.56; N, 24.6.

9-(4'-Cyanobutyl)-adenine (XVII).—This was prepared as described for XIV. Recrystallization from chloroform with the aid of decolorizing carbon gave 54% of white needles, m.p. 156–157°; $\nu_{max.}^{KBr}$ 3300 (NH); 2240 (C≡N); 1670, 1600, 1575 cm.$^{-1}$ (NH, C=C, C=N).

Anal.—Calcd. for $C_{10}H_{12}N_6$: C, 55.5; H, 5.62; N, 38.9. Found: C, 55.3; H, 5.52; N, 38.9.

9 - (p - Carbomethoxybenzyl) - 6 - mercaptopurine (XXII).—A mixture of 0.302 Gm. (1 mmole) of X, 15 ml. of methanol, and 0.076 Gm. (1 mmole) of thiourea was refluxed with stirring for 2 hours. During this time the solid nearly dissolved, then the product separated. The cooled mixture was filtered and the product washed with 20 ml. of cold water; yield, 270 mg. (90%), m.p. 289–290°, that was suitable for further transformation. Recrystallization from 2-methoxyethanol gave 200 mg. (67%) of pure product as pale-buff crystals, m.p. 290–291°; $\nu_{max.}^{KBr}$ 1710 (ester C=O); 1600, 1575, 1530 (C=C, C=N); 1275 cm.$^{-1}$ (ester C—O—C).

Anal.—Calcd. for $C_{14}H_{12}N_4O_2S$: C, 56.0; H, 4.03; N, 18.7. Found: C, 56.2; H, 4.16; N, 18.5.

9-(4'-Cyanobutyl)-6-mercaptopurine (XXIV).—This was prepared as described for XXII; yield, 200 mg. (86%), m.p. 284–285°. Recrystallization from ethanol gave pale-colored needles, m.p. 286–287°; $\nu_{max.}^{KBr}$ 2255 (C≡N); 1600, 1540 cm.$^{-1}$ (C=N, C=C).

Anal.—Calcd. for $C_{10}H_{11}N_5S$: C, 51.5; H, 4.77; N, 30.0. Found: C, 51.7; H, 4.90; N, 30.0.

9-(p-Carboxybenzyl)-adenine (XVI) Hydrochloride.—A mixture of 150 mg. (0.53 mmole) of XIV and 15 ml. of 1 *N* aqueous sodium hydroxide was refluxed for 45 minutes; the ester rapidly dissolved. The cooled solution was acidified to pH 2 with 6 *N* hydrochloric acid. The white solid was collected on a filter, washed with water, and dried at 100°; yield, 130 mg. (80%), m.p. 348–349°. Recrystallization from absolute ethanol gave analytically pure material, m.p. 350°; $\nu_{max.}^{KBr}$ 3450 (NH); 1700 (acid C=O, C=NH$^+$); 1600, 1510 cm.$^{-1}$ (NH, C=C, C=N).

Anal.—Calcd. for $C_{13}H_{12}ClN_5O_2$: C, 51.1; H, 3.97; N, 22.8. Found: C, 51.3; H, 4.15; N, 22.8.

9-(p-Carboxybenzyl)-6-mercaptopurine (XXIII).—From 150 mg. (0.50 mmole) of XXII, as described for the preparation of XVI, was obtained after recrystallization from 2-methoxyethanol 100 mg. (70%) of product, m.p. 318–320°; $\nu_{max.}^{KBr}$ 1685 (carboxyl C=O); 1595, 1570, 1525 cm.$^{-1}$ (C=N, C=C).

Anal.—Calcd. for $C_{13}H_{10}N_4O_2S$: C, 54.5; H, 3.57; N, 19.6. Found: C, 54.5; H, 3.70; N, 19.7.

9-(p-Carbomethoxybenzyl)-hypoxanthine (XX).—A solution of 500 mg. (1.65 mmoles) of X in 50 ml. of methanol and 5 ml. of 1 *N* aqueous hydrochloric acid was refluxed for 3.5 hours. Most of the solvent was spin evaporated *in vacuo*, then 25 ml. of water was added. The product was collected on a filter and recrystallized from methanol; yield, 350 mg. (75%) of white crystals, m.p. 288°; $\nu_{max.}^{KBr}$ 1715 (ester C=O); 1600, 1555 (C=O, C=N, C=C); 1280 cm.$^{-1}$ (ester C—O—C).

Anal.—Calcd. for $C_{14}H_{12}N_4O_3$: C, 59.1; H, 4.27; N, 19.7. Found: C, 58.9; H, 4.06; N, 19.5.

9-(p-Carboxybenzyl)-hypoxanthine (XIX).—A solution of 151 mg. (0.50 mmole) of X in 7 ml. of concentrated hydrochloric acid was refluxed for 2.5 hours with magnetic stirring during which time the product separated. The mixture was diluted with 15 ml. of water and cooled. The product was collected on a filter and washed with water; yield, 120 mg. (89%), m.p. 324–327°. Recrystallization from 2-methoxyethanol gave white crystals, m.p. 330–331°; $\nu_{max.}^{KBr}$ 1670 (carboxyl C=O); 1600, 1580 cm.$^{-1}$ (C=O, C=C, C=N).

Anal.—Calcd. for $C_{13}H_{10}N_4O_3$: C, 57.8; H, 3.77; N, 20.7. Found: C, 57.5; H, 3.91; N, 20.9.

9-(4'-Carboxybutyl)-hypoxanthine (XXI).—Hydrolysis of 235 mg. (1 mmole) of XI, as described for the preparation of XIX gave, after recrystallization from 2-methoxyethanol, 150 mg. (64%) of product, m.p. 260–261°; $\nu_{max.}^{KBr}$ 1690 (carboxyl C=O); 1650, 1600 (C=C, C=N, C=O).

Anal.—Calcd. for $C_{10}H_{12}N_4O_3$: C, 50.9; H, 5.14; N, 23.8. Found: C, 50.9; H, 5.22; N, 23.9.

9-(4'-Carboxybutyl)-adenine (XVIII).—Hydrolysis of 216 mg. (1 mmole) of XVII with concentrated hydrochloric acid for 3 hours, as described for the preparation of XIX, gave 190 mg. (81%) of product, m.p. 267–270°. Recrystallization from 2-methoxyethanol gave 140 mg. (60%) of white crystals, m.p. 272–273°.

Anal.—Calcd. for $C_{10}H_{13}N_5O_2$: C, 51.1; H, 5.58; N, 29.8. Found: C, 51.0; H, 5.63; N, 30.0.

9-(4'-Carboxybutyl)-6-mercaptopurine (XXV).—A mixture of 233 mg. (1 mmole) of XXIV and 10 ml. of concentrated hydrochloric acid was refluxed for 6 hours, then cooled and diluted with 25 ml. of ice water. The crude product was collected on a filter and washed with water, then dissolved in excess 5% aqueous sodium bicarbonate. Acidification gave 90 mg. (36%) of tan crystals, m.p. 257–258°. Recrystallization from 2-methoxyethanol gave an analytical sample, m.p. 258–259°; $\nu_{max.}^{KBr}$ 1710 (carboxyl C=O); 1600, 1575, 1545 cm.$^{-1}$ (C=C, C=N).

Anal.—Calcd. for $C_{10}H_{12}N_4O_2S$: C, 47.7; H, 4.82; N, 22.2. Found: C, 47.7; H, 4.90; N, 22.1.

Results

By plotting Vo/V against I for four or more concentrations of I, the concentration of I necessary to give 50% inhibition ($Vo/V_I = 2$) was readily determined, where Vo = velocity of the enzymic reaction with no inhibitor, V_I = velocity of reaction in the presence of inhibitor, and I = concentration of inhibitor (17). At least two of the points should be in the 30–70% inhibition range where the least error occurs. In a few cases where a saturated solution gave only 35–49% inhibition, such as with compound XX, the plot was extended to Vo/V = 2 to determine the concentration of I that would

TABLE I.—50% INHIBITION OF LDH AND GDH BY NUCLEOTIDE ANALOGS

Group	Compound[a]	mM Concentration for 50% Inhibition LDH	GDH
A	AMP	6.7	35
	IMP	67	66
B	Carboxybenzyl-Ad (XVI)	15	27
	Carboxybenzyl-Hx (XIX)	22	64
C	Carboxybutyl-Ad (XVIII)	30	40
	Carboxybutyl-Hx (XXI)	82	D[c]
	Valerate	340	210
D	Benzoate	27	95
	p-Toluate	24	
	{Adenosine / Benzoate}[b]	17	B[c]

[a] For abbreviations see *Footnote 1*. [b] Equal concentrations of both compounds. [c] Compound not sufficiently soluble to determine 50% inhibition. See this group in Table II for partial inhibition data.

give 50% inhibition. These 50% inhibition values for LDH and GDH are listed in Table I.

In cases where a compound gave less than 35% inhibition with a saturated solution, a group run was made for simultaneous comparison of two to four compounds at the same concentration (Table II); if one of the compounds in the group did give a normal 50% inhibition plot, the inhibition at lower concentration was taken from Vo/V_I *versus* I plot–for example compound XVIII in Table II, group C.

DISCUSSION

Both riboflavin-5′-sulfate and adenosine-5′-sulfate have been observed to be flavin-adenine dinucleotide inhibitors in the D-amino acid oxidase system (31, 32). In addition, AMP, IMP, and adenosine have been found to be inhibitors in alcohol dehydrogenase systems, whereas nicotinamide mononucleotide was not (33, 34). ADP-ribose, an hydrolysis product of DPN is a strong competitive inhibitor of LDH (35) and alcohol dehydrogenase (36). Therefore it was not unreasonable to expect that AMP would be an inhibitor in the LDH and GDH systems.

That AMP was an inhibitor in both the LDH and GDH systems is shown in Table I. Attempts to show that AMP was a "competitive" DPN inhibitor by Lineweaver-Burk plots were not conclusive since results varied between "competitive" and "partially competitive" (20, 37). Therefore, we chose to rely on molecular changes in inhibitor structure to determine whether the synthetic compounds were binding to the enzymes in the same manner as AMP, an approach which Reiner (38) considers to be more definitive and with which we concur (17). Thus, both the LDH and GDH systems could be used to search for chemical groupings that simulate the 5′-phosphoribosyl moiety of AMP as an inhibitor.

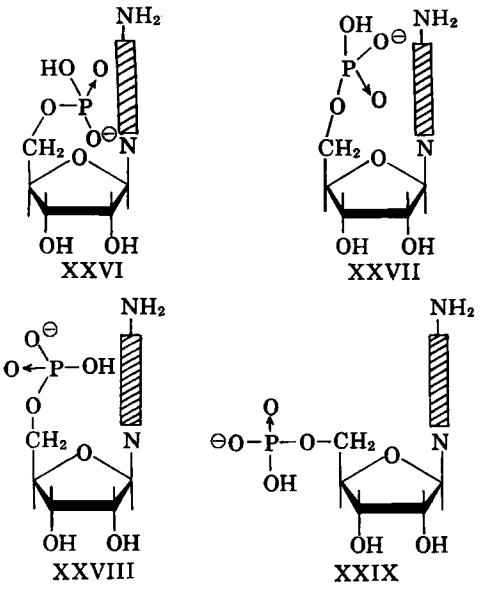
To check on the mode of binding of AMP to LDH and GDH, IMP and adenosine were measured as inhibitors. IMP inhibited both LDH and GDH, but considerably higher concentrations than AMP were needed for 50% inhibition (Table I). It can be concluded that the adenine moiety is necessary for binding to both enzymes, and the hypoxanthine moiety binds less effectively, probably because it is less basic. Adenosine was not sufficiently soluble to determine the concentration necessary for 50% inhibition. However, 7 mM adenosine gave no measurable inhibition of LDH, contrasted to AMP at 6.7 mM which gave 50% inhibition (Table I). Similarly, 15 mM adenosine gave only 7% inhibition of GDH, whereas AMP at 15 mM gave 29% inhibition (Table II, group C). Therefore it can be concluded that the phosphate group of AMP is necessary for binding to LDH and GDH when AMP acts as an inhibitor.

In the AMP structure, the purine ring has a rigid structure and the furanose ring can vary only slightly in conformation because of its slight pucker. The main possible changes in conformation of binding of AMP to an enzyme is because of the free rotation of the 1′-9 bond and all of the bonds of the phosphomethylene group. Examination of Leybold

TABLE II.—PARTIAL INHIBITION OF LDH AND GDH BY NUCLEOTIDE ANALOGS

Group	Enzyme	Compound[a]	mM Concn.	Vo/V_I	Inhibition, %
A	LDH	Valerate	18	1.15	13
		{Adenosine / Valerate}[b]	18	1.19	16
		Carboxybutyl-Ad (XVIII)	18	1.60	38
B	GDH	Benzoate	30	1.12	11
		AMP-2′(3′)	30	1.12	11
		{Benzoate / AMP-2′(3′)}[b]	30	1.16	16
		Carboxybenzyl-Ad (XVI)	30	2.30	67
		AMP	30	1.88	47
C	GDH	Valerate	15	1.00	0
		Adenosine	15	1.07	7
		{Adenosine / Valerate}[b]	15[b]	1.12	11
		Carboxybutyl-Ad (XVIII)	15	1.35	26
		AMP	15	1.40	29
D	GDH	Carboxybutyl-Ad (XVIII)	52	2.30	67
		Carboxybutyl-Hx (XXI)	52	1.23	19

[a] For abbreviations see *Footnote 1*. [b] Equal concentrations of both compounds.



models shows that there are two conformations of AMP with extremes in the distance of the P–O$^{\ominus}$ from the 6-amino group of AMP[3], 13.0 Å. (XXIX) and 3.0 Å. (XXVII). Another way of measuring the relative conformation is the distance between the P–O$^{\ominus}$ and the 9-nitrogen; in the case of XXIX, this distance[3] is 9.0 Å. Another type of extreme conformation is XXVI. In this case the P–O$^{\ominus}$ is tucked in towards the 9-nitrogen and the two atoms can almost touch. The latter conformation for enzyme binding is highly improbable because of the crowding of these ionic binding groups and probable steric hindrance to ionic binding to the enzyme. However, if the P–O$^{\ominus}$ and 9-nitrogen were separated about 3 Å., this type of conformation would be possible. One of the many possible intermediate conformations is shown by structure XXVIII.

Two side chains were selected for investigation to determine whether the 5′-phosphoribose moiety of AMP could be simulated in the binding of AMP to GDH or LDH or both. 9-(*p*-Carboxybenzyl)-adenine (XVI) has a fixed relationship between the 9-nitrogen of the adenine moiety and the charge of the $\begin{matrix} O \\ \diagdown \\ \diagup \\ O \end{matrix}\ominus$ of 8.6°. This distance compares favorably to that of 9.0 Å. between the O$^{\ominus}$ and 9-N of AMP[3] for the most extended conformation of AMP (XXIX). Slight rotation of the phosphate of XXIX group can bring this distance down to 8.6 Å. The most extended conformation of AMP and XVI both have a distance between the nitrogen of the 6-amino group[3] and the anionic group of 13 Å. All of the *p*-carboxybenzyl group except one hydrogen fits within the space occupied by the 5′-phosphoribosyl group. Thus XVI has the binding potential of the most extended conformation of AMP.

The second compound chosen was (4′-carboxybutyl)-adenine (XVIII). This compound has a flexible side chain which can vary in conformation from nearly the most extended conformation of AMP (XXIX) to nearly the least extended (XXVII) and include other conformations such as XXVI and XXVIII. In most (if not all cases) the bulk of the carboxybutyl side chain fits within the space occupied by the ribose-5′-phosphate moiety.

To determine whether 9-(*p*-carboxybenzyl)-adenine (XVI) and 9-(4′-carboxybutyl)-adenine (XVIII) simulate the inhibitor properties of AMP for LDH and GDH, three criteria must be met:

1. The compound should inhibit the same magnitude as AMP.
2. The hypoxanthine analog must inhibit less effectively than the adenine analog.
3. A given concentration of a compound such as XVI with two ionic points of attachment should show considerably greater inhibition than a solution of equal concentration of both of its moieties with one ionic point of attachment, such as benzoate and adenine. Actually adenosine and AMP-2′(3′) were used in place of adenine since the latter was not sufficiently soluble and has an undesirable acidic NH.

9-(*p*-Carboxybenzyl)-adenine (XVI) was an inhibitor of both LDH and GDH (Table I, group B) but inhibited in different ways. In the case of GDH, all three criteria for simulating AMP were met. First, XVI inhibited GDH slightly better than AMP. Second, 9-(*p*-carboxybenzyl)hypoxanthine (XIX) was required at 2.4 times the concentration of XVI to give 50% inhibition (Table I, group B); IMP was required in 1.9 times the concentration of AMP to give 50% inhibition, a favorable comparison. Third, 30 m*M* each of benzoate and AMP-2′(3′) in the same solution gave only 16% inhibition, whereas 30 m*M* of XVI gave 67% inhibition. The simulation of the inhibition of AMP by XVI is evidence that AMP has the fully extended conformation of the 5′-phosphoribosyl moiety when AMP binds to GDH, as discussed earlier.

With LDH, the criteria for simulating AMP were not met by 9-(*p*-carboxybenzyl)-adenine (XVI). First, the compound inhibited LDH half as well as did AMP (Table I). However, at only 1.5 times the concentration of XVI, 9-(*p*-carboxybenzyl)-hypoxanthine (XIX) gave 50% inhibition; this was far too inhibitory since there is a tenfold difference in concentration between AMP and IMP necessary for 50% inhibition. The reason for the high inhibition of XIX is apparent when one notes that 24 m*M* *p*-toluate gives 50% inhibition and benzoate at 27 m*M* (Table I); apparently XIX binds primarily as a derivative of benzoate and XIX has no second ionic binding point as required in the simulation of AMP. In addition, the third criterion was not met, since 17 m*M* concentrations of benzoate and adenosine in the same solution gave the same inhibition as 15 m*M* of XVI (Table I, group D). Apparently, XVI does not bind in a two ionic point attachment to LDH, but the adenine moiety of one molecule and the carboxybenzyl moiety of another molecule bind to one enzymic active site. Since the binding of AMP to LDH cannot be simulated by XVI, it can be concluded that AMP most probably does not have the most extended conformation of its ribose phosphate moiety when bound to LDH.

9-(4′-Carboxybutyl)-adenine (XVIII) was an

[3] Distances measured from the center of the O$^{\ominus}$ and N atoms.

inhibitor for both enzymes (Table I), and in both cases XVIII met the three criteria for simulating AMP. With GDH, XVIII gave 50% inhibition at only a slightly higher concentration than that required for AMP. The hypoxanthine analog (XXI) was not sufficiently soluble to determine the 50% inhibition point; therefore the equal concentration comparison listed in Table II, group D was made. At the highest obtainable concentration (52 m*M*), the hypoxanthine analog (XXI) gave 19% inhibition, whereas 9-(4′-carboxybutyl)-adenine (XVIII) gave 67% inhibition at 52 m*M*, thus meeting the second criterion. Third, in Table II, group C, 15 m*M* each of a mixture of valerate and adenosine gave only 11% inhibition, whereas 15 m*M* 9-(4′-carboxybutyl)-adenine (XVIII) gave 26% inhibition and 15 m*M* AMP gave 29% inhibition.

With LDH, a 30 m*M* concentration of the adenine analog (XVIII) was needed to give 50% inhibition, compared to 6.7 m*M* AMP. The other two criteria for simulation of AMP by XVIII were also met. As shown in Table I, group C, the AMP analog (XVIII) showed 50% inhibition at about one-third the concentration of the IMP analog (XXI). XVIII and XXI do not show the tenfold spread in concentration shown by AMP and IMP, for 50% inhibition of LDH is apparently due to appreciable one ionic-point attachment of the valerate side chain at the high concentration (82 m*M*) necessary for 50% inhibition. As shown in Table II, group A, 9-(4′-carboxybutyl)-adenine (XVIII) shows considerably higher inhibition than a solution of equal concentrations of valerate and adenosine.

In view of the reference material at the beginning of the *Discussion*, the data obtained in this paper, and the similarity in structure between AMP and half of the DPN molecule, it is probable that AMP binds to LDH and GDH in the same part of the active site as the AMP half of DPN. It follows that DPN probably has two different conformations when bound to LDH and GDH. The evidence is strong that 9-(4′-carboxybutyl)-adenine (XVIII) can simulate either conformation that AMP assumes when bound to these two different enzymes; it is probable that most of the conformations that AMP can assume when bound to other enzymes can be simulated by XVIII.

Side chains in addition to the *p*-carboxybenzyl group with a fixed conformation—or a limited locus of conformations—should be more specific as inhibitors when they simulate AMP. Of course these arguments can also apply to nucleotide substrates of purine and pyrimidine bases. One could even speculate that substrate-identical enzymes from different tissues might be expected to have different conformational requirements for the substrate.

In our opinion, this pilot study on simulation of the inhibitor properties of AMP against LDH and GDH by adenine derivatives substituted with an anionic side chain has been sufficiently definitive to warrant further study of these compounds including the mercaptopurines (XXIII, XXV) as inhibitors of enzymes interconverting nucleotides. By blocking the carboxylate group, a variety of possibilities for potentiation are possible to aid transport and membrane permeability *in vivo*, if necessary.

The search for other anionic side chains, particularly with a limited locus of conformational possibilities, attached to purines and pyrimidines that may inhibit biosynthesis of nucleic acid precursors is also warranted.

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Solution Phase Interaction of Nicotinamide with Ascorbic Acid

By DAVID E. GUTTMAN and DANA BROOKE†

A study was made of what appears to be a charge-transfer interaction that is responsible for the instantaneous generation of a yellow color in aqueous solutions containing nicotinamide and ascorbic acid. Spectral studies were utilized to elucidate some of the factors that influence the interaction. Analysis, by the method of continuous variation, showed that a 1:1 complex was formed. The extent of association was pH dependent and exhibited a maximum at a pH of approximately 3.8. The pH dependency was compared with that of the 3-carbamyl-1-methyl-pyridinium chloride–ascorbic acid system and indicated that the interactants were protonated nicotinamide and ascorbate anion. Association constants, at a number of different temperatures, were determined by using the Benesi-Hildebrand treatment of spectral data. The standard enthalpy change that resulted from association was estimated to be −1,500 cal.

COMPLEX OR ADDITION compound formation in systems containing ascorbic acid and nicotinamide was first reported in 1944 by Milhorat (1). Since that time a limited number of publications have appeared which have described in greater or lesser detail some of the properties of this combination. Bailey, Bright, and Jasper (2), for example, constructed temperature-composition diagrams for nicotinamide-ascorbic acid and nicotinic acid–ascorbic acid systems and demonstrated in this way that the reaction products had 1:1 stoichiometric compositions. Molecular weight determinations revealed that a high degree of dissociation occurred in aqueous and alcoholic solutions. Wenner (3) also studied the reaction and observed that it proceeded at a perceptible rate. His observation contrasts with those of others who reported that an almost instantaneous formation occurred upon mixing the interactants. Najer and Guépet (4), on the basis of studies in the U.V. and I.R. regions, concluded that the isolatable product was essentially the ascorbate of nicotinamide, but that some interaction occurred between the amide grouping and a secondary hydroxyl group of the acid. Investigations of the antiscorbutic properties of the complex were conducted and reported by Chalopin (5) and Nigeon-Dureuil (6). In addition, at least two patents were issued covering the product (7, 8).

The present investigation was undertaken to provide more definitive information about the nature of the interaction between the two vitamins, the extent of their association in aqueous solution, and the factors that influence such an occurrence. As will be seen, finite concentrations of a 1:1 complex were formed when the vitamins were combined in solution. The interaction was markedly dependent on the pH of the solution and appeared to be because of a charge-transfer interaction between protonated nicotinamide and ascorbate ion.

EXPERIMENTAL

Reagents.—Nicotinamide and ascorbic acid were U.S.P. grade. 3-Carbamyl-1-methyl-pyridinium chloride was synthesized by the method of Huff and Perlzweig (9), m.p. 237–239° (uncorrected). All solutions were freshly prepared in double-distilled, deionized water that had been thoroughly flushed with nitrogen. Disodium ethylenediaminetetraacetic acid (0.1%) was added to all solutions. Precautions were taken in all mixing procedures to prevent the

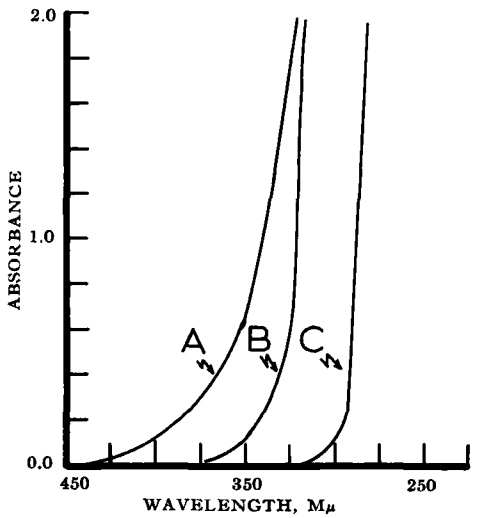

Fig. 1.—A plot showing the spectral characteristics of nicotinamide, ascorbic acid, and a mixture of the two. Curve A is the spectrum of a solution that was 0.02 *M* with respect to nicotinamide and 1.8 *M* with respect to ascorbic acid. Curve B is the spectrum of a 1.8 *M* solution of ascorbic acid. Curve C is the spectrum of a 0.02 *M* solution of nicotinamide.

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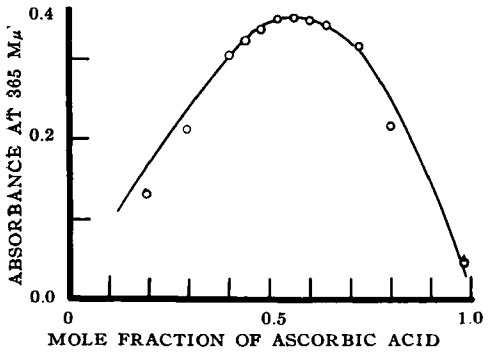

Fig. 2.—A plot illustrating the method of continuous variation used to investigate the stoichiometry of the ascorbic acid–nicotinamide interaction. The total solute concentration was 0.4 *M*.

introduction of air. Such precautions were necessary to preclude rapid autoxidation of ascorbic acid.

Methods.—Spectral studies were based on the fact that the addition of ascorbic acid to solutions of nicotinamide or its N^1-methyl derivative resulted in the instantaneous generation of a faint yellow color. Spectrophotometric examination, which is illustrated in Fig. 1, revealed the appearance of a new band as a long tail to the longer wavelength side of the ascorbic acid spectrum. Absorbance values of solutions were determined at two arbitrarily chosen wavelengths—345 and 365 mμ—and these served as quantitative indications of the concentration of complex in solution. The absorbance of each sample containing ascorbic acid and nicotinamide was corrected for the absorbance contribution of ascorbic acid by using as a solvent blank an ascorbic acid solution having the same concentration as the sample. This contribution was found to be small at lower pH values but became increasingly significant as the pH was increased. All spectrophotometric determinations were made with a Beckman model DU spectrophotometer, which was equipped with thermostatic plates, through which constant-temperature water was circulated to maintain the cell compartment at the desired temperature. Prior to absorbance measurements, the solutions were cooled or heated in an external bath to a temperature close to that of the compartment.

Analysis by Continuous Variation.—Equimolar solutions (0.4 *M*) of ascorbic acid and nicotinamide were prepared. Aliquots of the two solutions were mixed in various proportions to yield mixtures having a total volume of 25 ml. The absorbance at 365 mμ was determined for each solution using an appropriate solvent blank.

Influence of pH.—One-milliliter aliquots of a stock solution of nicotinamide or its N^1-methyl derivative were placed in a series of 25-ml. volumetric flasks. A 20-ml. volume of a 1.25 *M* solution of ascorbic acid was added to each flask. Volumes of concentrated aqueous sodium hydroxide were added; each flask was made up to volume with water. Absorbance values were determined at 365 mμ using solutions prepared in the same manner as solvent blanks, but which did not contain the amide. The pH of each solution was determined with a Beckman model G pH meter immediately after spectrophotometric examination.

Association Constant Determination.—One milliliter of a stock solution of nicotinamide was placed in each of a series of 10-ml. volumetric flasks. A volume of a 1.8 *M* solution of ascorbic acid, which had previously been adjusted to a pH of 4.0 by the addition of aqueous sodium hydroxide, was placed in each flask. Solutions were then made up to volume by the addition of water. An appropriate blank was formulated for each sample. Absorbances at 365 and 345 mμ were determined for each solution. Initially, the pH of each solution was measured. Repeated trials showed that dilution did not markedly change the pH from that of the original stock solution.

RESULTS

The analyses by continuous variation experiments are summarized in Fig. 2. The maximum absorbance appeared to correspond to a solution that was approximately equimolar with respect to the two vitamins and thus indicated the reversible formation of a complex of 1:1 stoichiometry. The high degree of dissociation of the complex was suggested by the absence of a well defined maximum in Fig. 2.

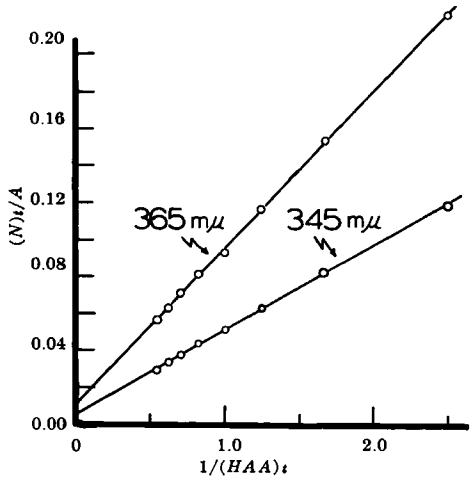

Fig 3.—A plot illustrating the Benesi Hildebrand treatment used to investigate the interaction between ascorbic acid and nicotinamide at 25°C. and at a pH of 4.0. The nicotinamide concentration was 0.04 *M*.

The stoichiometry was substantially confirmed by the plots shown in Fig. 3, where the effect of ascorbic acid concentration on the absorbances of solutions containing the two vitamins at constant pH was summarized. The treatment illustrated by the figure is analogous to that first proposed by Benesi and Hildebrand (10) and which is commonly used to determine complex association constants from spectral data. Here it was assumed that nicotinamide and ascorbic acid reversibly interacted to form a 1:1 complex, that the interaction obeyed the law of mass action, that the absorbance at the chosen wavelength was entirely due to the complex and followed Beer's law, and that the concentration of complex was insignificantly small compared to that of ascorbic acid. With these assumptions, it can be shown that

$$(N)_t/A = 1/a_c + 1/a_cK(HAA)_t \quad \text{(Eq. 1)}$$

where $(N)_t$ = analytical concentration of nicotinamide, A = absorbance at a particular wavelength, a_c = molar absorptivity of the complex at that wavelength, K = apparent association constant, and $(HAA)_t$ = analytical concentration of ascorbic acid.

Equation 1 predicts a linear relationship between $(N)_t/A$ and $1/(HAA)_t$ with a slope value of $1/a_cK$ and an intercept value of $1/a_c$. Excellent linearity of the curves of Fig. 3, over a wide range of ascorbic acid concentrations and at two different wavelengths, served to confirm the assumptions that were basic to the derivation of Eq. 1. Similar linearity was observed at three other temperatures.

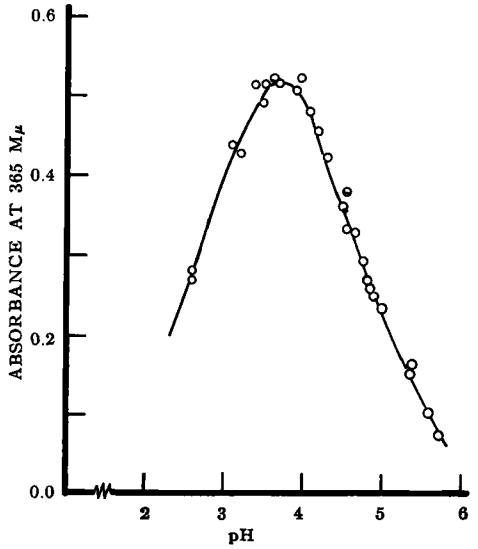

Fig. 4.—A plot illustrating the effect of pH variation on the interaction between ascorbic acid and nicotinamide at room temperature. Each solution was 0.05 M with respect to nicotinamide and 1.0 M with respect to ascorbic acid.

The influence of pH on the system and on a similar system containing 3-carbamyl-1-methyl-pyridinium chloride is depicted in Figs. 4 and 5. Here absorbance values of solutions of identical composition, except for hydronium-ion concentration, were plotted as a function of pH. The ascorbic acid–nicotinamide system exhibited the maximum degree of interaction at a pH of approximately 3.8. In the case of the N^1-methyl derivative, the degree of interaction increased with an increase in pH in a manner that is characteristic of the degree of ionization behavior of a weak acid. It will be seen that these results could be reasonably interpreted to mean that the species that underwent association to form the complex were ascorbate anion and the quaternary form of the pyridine derivative.

DISCUSSION

In the pH region which was investigated in the study illustrated by Fig. 4, nicotinamide existed in solution as the free base (N) and/or as the protonated base (NH^+). Similarly, ascorbic acid was present as the free acid (HAA) and/or as ascorbate ion (AA^-). The relative distribution of species in a particular solution would, of course, be dependent on the pH of that solution. This consideration leads to the possibility that one or more of four equilibria were operative and responsible for the formation of a colored 1:1 complex. These include

$$NH^+ + HAA = NH^+{:}HAA \quad \text{(Eq. 2)}$$

$$NH^+ + AA^- = NH^+{:}AA^- \quad \text{(Eq. 3)}$$

$$N + HAA = N{:}HAA \quad \text{(Eq. 4)}$$

$$N + AA^- = N{:}AA^- \quad \text{(Eq. 5)}$$

The pH profile of the interaction, as illustrated in Fig. 4, indicated, however, that equilibria of Eqs. 2 and 5 were not significantly responsible for the formation of a colored complex. It can be seen that at the pH extreme where the species NH^+ and HAA predominated and at the other extreme where N and AA^- predominated, the degree of interaction was low. This behavior can be verified visually by noting that the yellow color of a solution containing the vitamins can be instantaneously discharged by strong acidification or alkalinization. The pH of solutions which exhibited maximum absorbance and, therefore, maximum concentration of complex fell in the region where the interactants of equilibria of Eqs. 3 or 4 predominated.

The equilibrium expressed in Eq. 3 can be characterized by the association constant $K' = (NH^+{:}AA^-)/(NH^+)(AA^-)$, or in terms of the analytical concentrations of the vitamins as

$$K' = \left\{\frac{(NH^+{:}AA^-)}{(N)_t(HAA)_t}\right\}\left\{\frac{(H^+) + K_{NH^+}}{H^+}\right\}\left\{\frac{(H^+) + K_{HAA}}{K_{HAA}}\right\} \quad \text{(Eq. 6)}$$

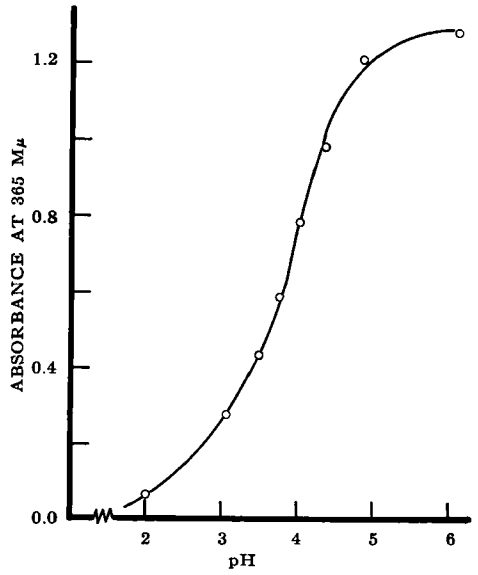

Fig. 5.—A plot illustrating the effect of pH variation on the interaction between ascorbic acid and N^1-methyl nicotinamide at room temperature. Each solution was 0.015 M with respect to N^1-methyl nicotinamide and 1.0 M with respect to ascorbic acid.

where K_{NH^+} and K_{HAA} = acidity constants of protonated nicotinamide and ascorbic acid, respectively.

Similarly, it can be shown that equilibrium of Eq. 4, involving free nicotinamide and free ascorbic acid, can be described by the association constant

$$K' = \left\{\frac{(N{:}HAA)}{(N)_f(HAA)_f}\right\}\left\{\frac{K_{NH^+} + (H^+)}{K_{NH^+}}\right\}\left\{\frac{K_{HAA} + (H^+)}{(H^+)}\right\} \quad \text{(Eq. 7)}$$

Differentiation of Eqs. 6 and 7 to obtain $d(\text{complex})/d(H^+)$, setting the resulting differentials equal to zero, and solving for the hydronium-ion concentrations at which the concentration of complex would be expected to maximize, yielded an identical result for both possible equilibria, that

$$(H^+)_{max.} = (K_{NH^+} \cdot K_{HAA})^{1/2}$$

or

$$pH_{max.} = {}^1/_2 pK_{NH^+} + {}^1/_2 pK_{HAA} \quad \text{(Eq. 8)}$$

Using a value of 3.42 for the pKa of protonated nicotinamide (11) and that of 4.17 for ascorbic acid (12), the pH of maximum concentration of complex was calculated to be 3.80. The experimental curve exhibited a maximum which agreed closely with this theoretically expected value. The close agreement between theory and experiment confirmed that one or both of the equilibria were operative but did not permit a differentiation between the possibilities.

Since it was impossible to differentiate in this way between the equilibria which, on the one hand, involved the ionized, and on the other, the unionized forms of the vitamins, the influence of pH on a similar, previously unreported interaction between ascorbic acid and 3-carbamyl-1-methyl-pyridinium chloride was studied. Here, the degree of ionization of the N^1-methyl nicotinamide was pH independent. It was therefore felt that if a similar interaction occurred and was influenced by pH, then the nature of the influence would point to either unionized ascorbic acid or ascorbate ion and the cationic form of nicotinamide as the interacting species. Interaction did occur and the pH profile shown in Fig. 5 unequivocally implicated ascorbate ion as the species which was involved. This evidence, although indirect, strongly suggests that the predominant equilibrium which resulted in the generation of the yellow color in solutions of ascorbic acid and nicotinamide was that described by Eq. 3.

On the basis of this evidence, the association constants which were calculated from the Benesi-Hildebrand plots by regression analysis and which were based on the analytical concentrations of the vitamins were multiplied by the factor

$$\left\{\frac{(H^+) + K_{NH^+}}{(H^+)}\right\}\left\{\frac{(H^+) + K_{HAA}}{K_{HAA}}\right\}$$

to yield constants which were consistent with the implicated equilibrium. These are presented for the temperatures and wavelengths investigated in Table I. The effect of temperature on the equilibrium was summarized in Fig. 6, where log K' was plotted as a function of the reciprocal of absolute temperature. The enthalpy change which resulted from association was estimated from this graph to be −1,500 cal.

TABLE I.—ASSOCIATION CONSTANTS FOR THE COMPLEX FORMED BY THE INTERACTION OF NH^+ AND AA^- IN AQUEOUS SOLUTION

Temp., °C.	Wavelength Used for Determination, mμ	K' (with 95% confidence range), L./Mole
10	345	1.64 ± 0.038
21	345	1.52 ± 0.024
25	345	1.45 ± 0.035
25	365	1.43 ± 0.020
29	345	1.40 ± 0.020

A number of considerations indicate that charge-transfer forces were responsible for the formation of the ascorbic acid–nicotinamide complex in solution. That the interaction involved ascorbate ion and the cationic form of nicotinamide immediately suggests such a possibility because of the well-known ability of pyridinium compounds to function as electron acceptors in charge-transfer interactions with negatively charged species. In addition, the bathochromic shift which occurred as a manifestation of the interaction is similar to those observed in a number of charge-transfer systems. Furthermore, the association constants determined in the present study are of the same order of magnitude as those reported by Kosower (13) for the charge-transfer complexes of iodide and substituted pyridinium compounds. The observed enthalpy change is approximately the same as that reported by Negoro, *et al.* (14), for the interaction of nicotinamide with *p*-aminosalicylic acid which they attributed to a charge-transfer mechanism.

Evidence has been presented which permits a more quantitative understanding of the interaction between ascorbic acid and nicotinamide, an interaction which has been variously referred to as a pharmaceutical incompatibility and a means of providing a more efficient therapeutic combination. In view of the low association constant of the complex, and the fact that the physiological pH does not favor the interaction, it is doubtful that the activity of the vitamin combination is in any way altered from that of the individual components. In addition to its pharmaceutical significance, the interaction is of interest because it focuses attention on ascorbate

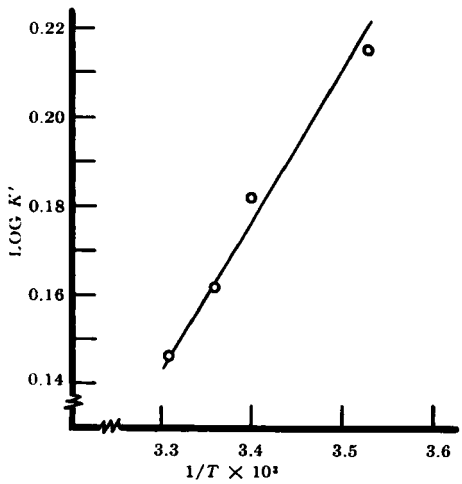

Fig. 6:—A plot illustrating the temperature dependency of the interaction between ascorbic acid and nicotinamide.

ion as a participant in a charge-transfer interaction and points to the possibility that it can interact similarly with enzyme or coenzyme systems in the body. It is conceivable that such an occurrence might be involved in metabolic processes which are dependent on this vitamin.

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Determination of Ephedrine Salts in Liquid Dosage Forms

By MARTIN I. BLAKE and DANIEL A. NONA

Analytical procedures are presented for the determination of ephedrine salts in syrups, elixirs, solutions, and injections. The ephedrine is extracted with the strong cation exchange resin, Dowex 50 X-8, and is subsequently eluted with 2 normal hydrochloric acid. After evaporation of the eluate to dryness, the residue is titrated nonaqueously with perchloric acid in dioxane in the presence of mercuric acetate. In formulations containing sodium chloride and other salts, the ephedrine is eluted with an alcoholic solution of ammonia. The eluate is aerated until all the ammonia is expelled. The ephedrine is determined by titration with hydrochloric acid. Comparison is made with the official assay for ephedrine sulfate solution.

EPHEDRINE, ephedrine salts, and their dosage forms have been official since U.S.P. XI and N.F. VI. The official assay procedure has usually involved ether extraction of the alkaloid liberated from the salt combination by addition of base. The ephedrine is then determined by residual alkalimetry. Ephedrine sulfate and phenobarbital capsules (1) are analyzed for ephedrine by a distillation method developed by Hilty (2) and further applied by Hilty and Wilson (3). Until recently ephedrine sulfate capsules (4) were also analyzed by this method. There is no official assay for N.F. XI ephedrine sulfate syrup.

Colorimetric procedures have been developed for the estimation of ephedrine. These have been reviewed by Higuchi and Bodin (5) and Snell and Snell (6).

Ephedrine and ephedrine salts have been determined by nonaqueous titration. Auerbach (7) titrated ephedrine in acetic acid with acetous perchloric acid. Salts of organic bases were titrated with perchloric acid in dioxane by Pifer and Wollish (8). Salts of alkaloids including ephedrine were studied. Titrations were effected visually and potentiometrically. Since chloride ion is too weakly basic to react quantitatively with the perchloric acid, the authors found that by adding mercuric acetate to the titration mixture, the chloride is tied up as undissociated mercuric chloride. The liberated acetate ion is then titratable with perchloric acid. Mercuric acetate itself is essentially undissociated in acetic acid and, therefore, does not titrate as a base. Rink and Lux (9) and Ekeblad (10) determined ephedrine hydrochloride with acetous perchloric acid by applying the method of Pifer and Wollish. "The British Pharmacopoeia" (11) recognizes this procedure for the assay of ephedrine hydrochloride tablets. Chatten and Pernarowski (12) analyzed for ephedrine in oily nasal sprays with acetous perchloric acid. Aqueous sprays were extracted with chloroform prior to nonaqueous titration.

Ion-exchange resins have been employed in the determination of alkaloidal salts including ephedrine hydrochloride and ephedrine sulfate. Amberlite IR-4B, a weak anion-exchange resin, was used by Jindra (13) for ephedrine sulfate but was found unsuitable (14) for ephedrine hydrochloride. The resin removes the acid component of the sul-

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fate salt while the alkaloidal base appears in the eluate which is titrated visually with standard hydrochloric acid. This resin apparently does not split the ephedrine hydrochloride quantitatively. Samuelson (15) has noted that with weakly basic anion exchangers incomplete conversion may occur when salts of fairly strong bases such as ephedrine are involved. Saunders, *et al.* (16), used a strong anion-exchange resin to liberate the free base from ephedrine hydrochloride. Since this resin is a salt splitter, inorganic salts interfere. These authors also noted that weak anion exchangers are unsuitable for salts of strong bases as ephedrine. However, Vincent, *et al.* (17), found Amberlite IR-45 (a weakly basic anion-exchange resin) suitable for the estimation of ephedrine sulfate in liquid dosage forms. The column was washed with water to remove sodium and other interfering components. The ephedrine was removed subsequently by elution with 75% ethanol.

Huyck (18) found that Amberlite IRC-50 was a good adsorbent for ephedrine alkaloid. Blaug and Zopf (19) used this resin to extract the ephedrine from an elixir which also contained an antihistamine salt. The ephedrine was retained by the resin while the antihistamine salt appeared in the eluate. Ephedrine salts were not employed in their study.

The present paper describes procedures for determining ephedrine salts in liquid dosage forms by ion-exchange chromatography and nonaqueous titrimetry. A modification is presented which eliminates interference by inorganic salts and certain synthetic sweetening agents.

EXPERIMENTAL

Preparation of Exchange Column

The strongly acidic cation-exchange resin Dowex 50 X-8 (200–400 mesh) was used in this study. A Mohr buret cut to a length of 30 cm. served as the chromatographic tube. The end of the tube was joined to a glass tip by a 5-cm. piece of rubber tubing. A Hoffman clamp was fixed to the rubber tubing midway between the buret and the glass tip. A plug of glass wool was inserted into the bottom of the buret to support the resin column. Ten grams of resin was added to the tube in the form of a suspension in water. When the resin settled completely, the column was washed with 200 ml. of distilled water, 100 ml. of 1 *N* hydrochloric acid, and finally with distilled water until the eluate was neutral. The column was ready for use. A layer of solvent was maintained above the resin column at all times.

Assay Procedure

Method A.—A 100-mg. sample of ephedrine sulfate or ephedrine hydrochloride, accurately weighed, was dissolved in 10 ml. of distilled water. The solution was added to the resin column. The column was washed with at least 100 ml. of distilled water. The ephedrine was then eluted from the column with 2 *N* hydrochloric acid. One-hundred milliliters of eluate was collected in a 150-ml. beaker. The solution was evaporated to dryness on a steam plate. Ten milliliters of distilled water was added; the solution was again evaporated to dryness. This was repeated once. The residue was analyzed for ephedrine content by nonaqueous titration.

Ten milliliters of glacial acetic acid, 20 ml. of dioxane, and 10 ml. of 6% mercuric acetate in glacial acetic acid were added to the beaker containing the ephedrine hydrochloride residue. The solution, magnetically stirred, was titrated with 0.1 *N* perchloric acid in dioxane using a Fisher titrimeter equipped with a calomel and glass electrode system.

Titration was also effected visually by adding two drops of a 0.2% methyl violet solution in glacial acetic acid to the titration beaker. The proper indicator color change was noted by using indicator solution in conjunction with a potentiometric titration. The color change corresponding to the graphic end point was from a violet to an intense blue coloration and was readily detectable with the addition of one drop of titrant at the end point.

Method B.—A sample of ephedrine hydrochloride or ephedrine sulfate was applied to the resin column as described under *Method A*. The ephedrine was eluted with 5% ammonium hydroxide in 95% alcohol prepared by diluting stronger ammonia water with ethanol. A total of 60 ml. of eluate was collected in a 150-ml. beaker. The solution was gently aerated by passing a stream of air over the beaker until the ammonia was completely expelled from the solution. A study, reported later in this paper, indicated that at least 3 or 4 hours were necessary for this purpose. The liquid level in the beaker was kept constant by adding alcohol from time to time. The solution was then titrated visually with 0.1 *N* hydrochloric acid using three drops of methyl red T.S. as the indicator.

Method C.—After titration of the eluate as de-

Table I.—Analysis of Ephedrine Salts, Free and in the Presence of Sodium Chloride

Ephedrine salt	Weighed, mg.	Sodium Chloride, mg.	Recovery, %	Analysis, Method
Ephedrine HCl	122.0	...	98.7	A
	123.4	...	99.7	A
	124.7	...	100.2	A
	126.2	...	99.2	A
	104.6	50.2	98.7	B
	...	...	99.2	C
	113.2	49.6	100.3	B
	...	...	98.2	C
	122.5	51.5	100.2	B
	...	...	100.7	C
Ephedrine sulfate	96.4	...	99.9	A
	102.7	...	99.2	A
	106.3	...	99.6	A
	110.0	...	99.7	A
	103.7	57.1	98.7	B
	...	...	98.2	C
	105.5	52.1	99.9	B
	...	...	100.1	C
	109.3	74.5	100.8	B
	...	...	102.1	C

TABLE II.—ANALYSIS OF LIQUID DOSAGE FORMS CONTAINING EPHEDRINE SULFATE

Dosage Form	Sample	Labeled Amount	Recovery, %	Method
Ephedrine sulfate solution	I (N.F.)	3 Gm./100 ml.	99.51 ± 1.05[a]	B
			100.37 ± 1.68	C
			99.00[b]	D
	II (N.F.)	3 Gm./100 ml.	101.46 ± 1.21	B
			100.00 ± 1.06	C
			103.33	D
	II	1 Gm./100 ml.	100.52 ± 0.33	B
			100.31 ± 0.47	C
			99.40	D
Ephedrine sulfate elixir	I	0.440 Gm./100 ml.	100.49 ± 0.24	B
			101.41 ± 1.01	C
			99.55	D
Ephedrine sulfate syrup	I	0.220 Gm./100 ml.	99.88 ± 1.02	A
			100.70 ± 0.84	B
			100.61 ± 1.17	C
			102.35	D
	I (N.F.)	0.400 Gm./100 ml.	99.60 ± 0.93	A
			97.96 ± 1.06	B
			98.35 ± 0.81	C
			98.79	D
	II	0.400 Gm./100 ml.	100.20 ± 0.94	B
			100.35 ± 0.55	C
			98.50	D
Ephedrine sulfate injection	I	50 mg./ml.	97.65 ± 0.41	B
			97.55 ± 1.25	C

[a] Standard deviation; based on at least five determinations. [b] Results of official assay are averages of duplicate runs.

scribed under *Method B*, the solution (now containing ephedrine hydrochloride) was evaporated to dryness and the residue analyzed by nonaqueous titration as described under *Method A*.

Method D.—Certain samples (indicated in Table II) were analyzed for comparative purposes by the procedure described for N.F. XI ephedrine sulfate solution.

For *Methods A*, *B*, and *C* blank runs were conducted with each series of four determinations, and the necessary corrections were made in the calculations. The data for the analysis of ephedrine salts, free and in the presence of sodium chloride, are shown in Table I.

Aeration Time

The time of aeration necessary for complete removal of ammonia from eluate obtained in *Method B* was determined in the following manner. A series of pure ephedrine samples was prepared by weighing accurately 75 to 100 mg. of pure alkaloid into 100-ml. beakers. Sixty milliliters of 5% ammonia in alcohol was added to each beaker. Each solution was subjected to gentle aeration by directing a fine stream of air above the surface of the liquid for varying intervals of time. The solutions were analyzed for ephedrine content by titration with 0.1 *N* hydrochloric acid using methyl red T.S. as the indicator.

Analysis of Liquid Dosage Forms

A number of dosage forms containing ephedrine salts were analyzed by the procedures previously described. For viscous solutions, as the syrup and elixir, an aliquot containing about 100 mg. of ephedrine salt was transferred by pipet to a suitable beaker. The solution was diluted with an equal volume of 50% alcohol and added to the ion-exchange column. When the last of the solution passed below the surface of the resin, the column was washed with 150–200 ml. of distilled water. The ephedrine was eluted from the column as described for *Methods A* or *B*. For the ephedrine sulfate solutions and the injection, a suitable aliquot was added directly to the column and treated as above. The data for the analysis of liquid dosage forms are shown in Table II. Samples designated as I are commercial preparations, those listed as II are preparations routinely prepared in the manufacturing pharmacy of our pharmacy department.

DISCUSSION

The official assay for ephedrine salts, free and in dosage forms, is based on an extraction procedure with ether. It is tedious and time consuming. While no assay is recognized for the syrup, application of the official assay for the solution to the syrup did yield favorable results (Table II).

The strong cation-exchange resin, Dowex 50 X-8, is an effective salt splitter and removes the ephedrine quantitatively from its salt form. Excellent results were obtained by *Method A* for both the hydrochloride and the sulfate. The data are reported in Table I. Since this resin is a salt splitter, sodium chloride interferes. Both sodium ion and ephedrine are retained by the column. Elution with 5% ammonium hydroxide in alcohol (*Method B*) displaces the ephedrine from the column but not the sodium. This proved to be an effective desalting technique. The eluate was aerated by passing a stream of air over the solution until all the ammonia was removed. The effect of aeration time on ephedrine recovery was noted experimentally. Under the conditions used, high results (122.6%), due to the presence of ammonia calculated as ephedrine, were still obtained after aeration for 2.5 hours. All the ammonia was removed by aeration for 3 hours, and there was no appreciable loss of ephedrine after aeration for 8 hours. Results obtained by *Method B* for ephedrine

salts in the presence of sodium chloride are recorded in Table I. The ephedrine hydrochloride formed in *Method B* was then determined by nonaqueous titration (*Method C*) as a confirmatory procedure.

The proposed methods were applied to liquid dosage forms including solutions, an elixir, syrups, and an injection. The data are reported in Table II. Since the solutions contain sodium chloride, *Method A* could not be used. Favorable results were obtained with *Methods B*, *C*, and *D*.

The elixir reported in Table II was a commercial sample and when analyzed by *Method A* gave very high results. The manufacturer upon request was kind enough to supply the formula of the elixir which did contain appreciable amounts of sodium saccharin and sodium chloride. Quantitative results were obtained by *Methods B* and *C*. The elixir responded to the official assay for ephedrine sulfate solution.

The syrups yielded good results by the proposed methods as well as by the official assay procedure for the solution. Since the injection contains sodium chloride, *Methods B* and *C* were used.

Coloring agents found in the elixir and syrup do not interfere. They are readily washed from the column with water. Nonionic agents and acidic components in general, are not adsorbed by the column. Where synthetic sweeteners such as sodium saccharin are used in the formulation, *Method B* must be employed. Other organic bases and their salts will interefere with the proposed methods. However, if the base strength differs significantly from that of ephedrine, a nonaqueous differential titration may be possible.

Since four assays may be conveniently conducted at the same time, the proposed methods are less time consuming and less tedious than the official assay. The procedures are simple, accurate, and applicable to all commonly available liquid dosage forms of ephedrine salts. They should also be applicable to solid dosage forms and to combinations of ephedrine with other therapeutic agents such as barbiturates.

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ALTHOUGH the oxidative route of degradation of ascorbic acid has been extremely well studied, relatively few papers have appeared dealing with degradative loss of ascorbic acid under anaerobic conditions. Since in practice reduction in the concentration of this vitamin in liquid pharmaceutical preparation appears to follow largely the latter route, serious study of the factors influencing its rate was felt needed. Even in instances where losses in the ascorbic acid concentration are of no major concern, the gas produced in the process often poses a problem.

Previous studies have been largely of qualitative nature. Reichstein and Grüssner (1), for example, showed that when ascorbic acid was heated with 0.2 *N* hydrochloric acid a decrease in the iodine consumption and furfural was formed. Their observation was part of a work on the synthesis of ascorbic acid from 2-keto-L-gulonic acid.

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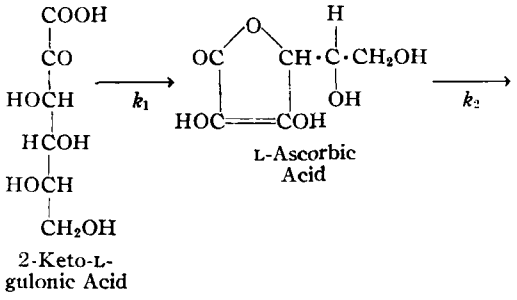
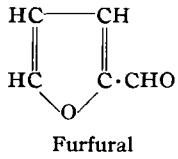
The enolization and lactonization of methyl 2-keto-L-gulonate into ascorbic acid could be brought about by sodium methylate and also by hydrochloric acid. Heating of 2-keto-L-gulonic acid with hydrochloric acid thus is presumed to lead to the consecutive reactions





Regna and Caldwell (2) have studied, again only in the strongly acid solution, the kinetics of this reaction and the kinetics of the transformation of some other 2-ketopolyhydroxy acids to their ascorbic acid analogs and the decomposition of these compounds. For the transformation of 2-keto-L-gulonic acid into L-ascorbic acid in 5 *M* hydrochloric acid at 59.9°, a first-order rate constant $k_1 = 2.53 \times 10^{-3}$ minutes^{-1} was found. For the decomposition of L-ascorbic acid into furfural under the same conditions, the first-order rate constant was 4.91×10^{-4} minutes^{-1}.

Taylor, Fowler, McGee, and Kenyon (3) noted that carbon dioxide is evolved when ascorbic acid was heated, again in 12% hydrochloric acid. They submitted kinetic data on the evolution of carbon dioxide from L-ascorbic acid and from D-glucuronic acid, D-galacturonic acid, pectic acid, alginic acid, celluronic acids, and oxidized starch. The first-order rate constant for the evalution of carbon dioxide from ascorbic acid was more than twice as great as that of its nearest neighbor in the series, D-galacturonic acid. This indicated the profound effect of an enediol structure on the stability of the adjacent lactonized carboxyl group. A rather rapid evolution of carbon dioxide occurs from ascorbic acid until approximately 28% by weight is lost, followed by slow evolution for many hours more, finally attaining a total value in excess of 30%. If only the carboxyl group supplied the carbon dioxide, the evolution should have been 25.0%. The results indicated that the carboxyl group decomposed rapidly and that there is a further slow decomposition evolving more carbon dioxide.

Von Euler and Hasselquist (4) found that by heating ascorbic acid with 1 *N* sodium hydroxide at 100° in nitrogen atmosphere the lactone ring was obviously opened and the salt of the corresponding hexuronic acid was formed.

For the anaerobic degradation of ascorbic acid acid at pH 1.10 Fischer-Jensen (5) found a first-order rate constant $k = 0.323$ hours^{-1} at 90° and $k = 0.801$ hours^{-1} at 100°, thus giving $Q_{10} = 2.5$. The decomposition was faster at pH 2.5 than at pH 6 and was independent of the ascorbic acid concentration.

By heating ascorbic acid solutions of different pH values below 5.5 under anaerobic conditions Cier, Nofre, and Drevon (6) showed that carbon dioxide, furfural, and xylose were formed. The rate of formation of only furfural was followed and was found to be very slow at pH 5 and higher; the rate, however, increased sharply by decreasing pH. The authors proposed the following scheme for the nonoxidative degradation of ascorbic acid in acid medium

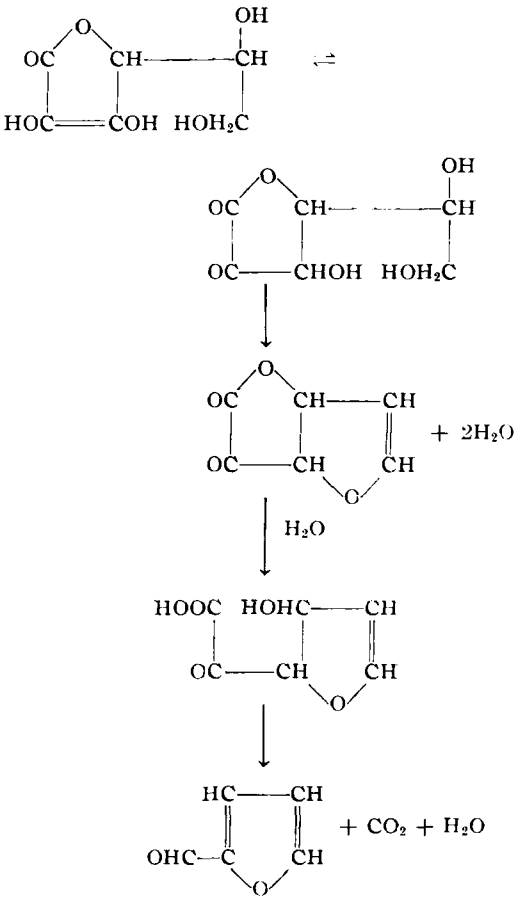


TABLE I.—COMPARISON OF RESULTS OBTAINED BY THE IODOMETRIC METHOD (A) AND BY THE DICHLOROPHENOL-INDOPHENOL METHOD (B) IN ANALYSIS OF ASCORBIC ACID SOLUTIONS OF DIFFERENT pH DEGRADED BY HEATING AT 96°C.

pH 1.08			pH 4.10			pH 5.20			pH 7.04			pH 10.40		
	Ascorbic Acid Concn. Found, $M/L. \times 10^3$			Ascorbic Acid Concn. Found, $M/L. \times 10^3$			Ascorbic Acid Concn. Found, $M/L. \times 10^3$			Ascorbic Acid Concn. Found, $M/L. \times 10^3$			Ascorbic Acid Concn. Found, $M/L. \times 10^3$	
Hrs. Heated	A	B	Hrs. Heated	A	B	Hrs. Heated	A	B	Hrs. Heated	A	B	Hrs. Heated	A	B
0	9.66	9.50	0	9.79	9.88	0	9.92	9.97	0	9.97	9.95	0	9.66	9.70
1	9.06	9.10	3	9.24	9.03	6	9.48	9.41	6	9.63	9.50	7	8.53	8.88
2	8.62	8.68	7.6	7.89	7.83	16.7	8.58	8.26	15	9.30	9.24	21	7.99	8.00
3.3	8.00	8.12	21.2	5.37	5.44	23.5	7.88	7.95	20	9.07	8.99	31.2	7.47	7.12
4.3	7.55	7.55	...	...	...	40.7	6.75	6.83	42.6	8.06	7.99	45	6.80	6.77
5.3	7.10	7.16	...	...	...	47.5	6.40	6.25	53	7.71	7.70	...	...	...
6	7.05	7.05	...	...	...	...	...	...	62	7.28	7.19	...	...	...
$t_{1/2}$	12.7 hr.	13 hr.		25 hr.	25 hr.		75 hr.	75 hr.		145 hr.	145 hr.		82 hr.	79 hr.

This scheme does not explain how the xylose is formed. The authors exclude the possibility of xylose being the precursor of furfural since xylose by heating in slightly acid medium only gives traces of furfural.

The present studies have been concerned with the exact rate of anaerobic degradation of ascorbic acid in acid and in alkaline medium and determination of the extent of catalysis by hydrogen and hydroxyl ions and by general acids and bases.

EXPERIMENTAL

Materials.—Ascorbic acid U.S.P. from Hoffmann-La Roche Co. was used. Iodometric titration of this product according to the method in U.S.P. XVI showed that it contained 99.85% $C_6H_8O_6$.

All reagents used were of analytical grade. The water used was distilled water redistilled from a neutral glass still, boiled and cooled under oxygen-free nitrogen.

Assay.—The residual ascorbic acid concentration of the heated solutions was determined iodometrically. An aliquot part of the sample, usually 5.00 ml., was acidified with diluted sulfuric acid and titrated with 0.01 *N* iodine using a few drops of starch T.S. as indicator.

In some cases the ascorbic acid concentration was also determined by the dichlorophenol-indophenol method given by U.S.P. XVI for ascorbic acid injection.

Degradation Studies.—A 176-mg. quantity (0.001 mole) of ascorbic acid was dissolved in 100 ml. of the appropriate buffer solution containing a sufficient amount of sodium chloride to give the ascorbic acid solution an ionic strength $\mu = 0.50$. The solution was filled into 5-ml. ampuls, and the air in the ampuls was replaced by nitrogen. The ampuls were sealed and heated at 96° in a constant temperature bath. At appropriate intervals ampuls were taken out from the bath, cooled on ice, and the solution analyzed.

Determination of pKa_1 and pKa_2 of Ascorbic Acid at 96° and Ionic Strength $\mu = 0.5$.—pKa_1 was determined by measuring the pH at 96° of solutions containing equal amounts of ascorbic acid (A) and monohydrogen ascorbate (A^-) and a sufficient amount of sodium chloride to give $\mu = 0.50$. $pKa_1 = 3.94$ was found.

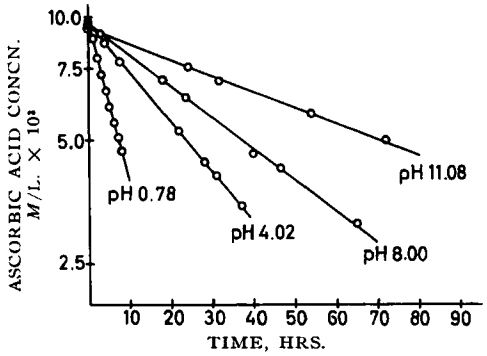


Fig. 1.—Plots showing the overall first-order character of the anaerobic degradation of ascorbic acid at different pH values and 96°C. For the actual runs 0.2 *N* perchloric acid (pH 0.78), 0.5 *M* acetate (pH 4.02), 0.15 *M* borate (pH 8.00), and 0.25 *N* NaOH (pH 11.08) were used.

TABLE II.—HALF-LIFE OF ASCORBIC ACID DEGRADATION AT 96°C. AND DIFFERENT pH VALUES AND DIFFERENT IONIC STRENGTH

Ionic Strength	Half-Life, hrs. pH 1.1	pH 4.0	pH 6.5	pH 10.2
0.1	13.2	31	140.3	35
0.2	13.1	30.5	139.8	35
0.3	...	30	139	36
0.4	12.9	30	...	36
0.5	...	30	137	36
0.6	13.1	...	...	...

pKa_2 was determined by measuring (under nitrogen) the pH at 96° of solutions prepared by mixing equal amounts of monohydrogen ascorbate (A^-) and ascorbate ($A^=$) and adding a sufficient amount of sodium chloride to give $\mu = 0.50$. The following equations were used for calculation of pKa_2

$$pKa_2 = pH - \log \frac{[A^-] - [OH^-]}{[A^-] + [OH^-]} \quad \text{(Eq. 1)}$$

$$\log [OH^-] = pH - 11.70 \quad \text{(Eq. 2)}$$

The relationship between pH and $[OH^-]$ given by Eq. 2 was obtained as the result of pH measurements in solutions of sodium hydroxide of known hydroxyl ion concentration at 96° and $\mu = 0.50$.

It became difficult to determine pKa_2 with great accuracy. The average value of several determinations was $pKa_2 = 10.5$.

RESULTS AND DISCUSSION

Comparison of the Results Obtained by the Iodometric Method and by the Dichlorophenol-Indophenol Method.—Solutions of different pH were heated at 96° and the residual ascorbic acid concentration determined at appropriate intervals according to the iodometric method and the dichlorphenol-indophenol method. The half life ($t_{1/2}$) was found graphically. Table I shows that the two methods gave essentially the same results.

Order of Reaction with Respect to Ascorbic Acid.—The rate of disappearance of ascorbic acid from the solutions was found to be first order with respect to ascorbic acid at pH values from 0.4 up to 11.4. In all cases there was a linear relationship between time and logarithm of residual ascorbic acid concentration as shown in Fig. 1. The absence of lag time strongly suggests that the effective mechanism contains only one rate-determining step involving loss of the reducing capacity of the solutions. If ascorbic acid is initially converted to some other form still capable of reacting with iodine or the indophenol, this step would have to be exceedingly rapid. Since other data suggest that the vitamin is relatively stable under these conditions, it is unlikely that any irreversible formation of a readily oxidizable species takes place to a significant extent.

Primary Salt Effect.—A series of runs were made keeping pH, ascorbic acid concentration, and buffer concentration constant in each series but varying the ionic strength by addition of different amounts of sodium chloride. Table II shows that there was no primary salt effect at pH 1.1, 4.0, 6.5, or 10.2.

Catalytic Effect of General Acids and Bases on the Anaerobic Degradation of Ascorbic Acid.—The catalytic effect of different buffers was determined by making runs with each buffer varying the total buffer concentration by keeping the pH and the ionic strength constant ($\mu = 0.50$). Straight lines were obtained by plotting the observed k values against buffer concentration, the slopes of which were a measure of the catalytic activity of the buffers.

Figure 2 shows that phosphate buffers with pH 2.14, 2.60, and 3.05 have a catalytic effect on the anaerobic degradation of ascorbic acid. At pH $\lesssim$ 2.60, more than 95% of the total ascorbic acid will exist in the undissociated form. It is therefore reasonable to assume that the curves representing the runs at pH 2.14 and pH 2.60 give a picture of the catalytic effect of H_3PO_4 and $H_2PO_4^-$ on the undissociated ascorbic acid. If we assume this, it is possible to calculate the catalytic constants $k_{H_3PO_4}$ and $k_{H_2PO_4^-}$ of H_3PO_4 and H_2PO_4, using the equations

$$k_{obs} = k_o + k_{H_3PO_4} \cdot [H_3PO_4] + k_{H_2PO_4} - [H_2PO_4^-] \quad \text{(Eq. 3)}$$

$$\frac{[H_2PO_4^-]}{C - [H_2PO_4]} = \frac{ka_1}{[H^+]} \quad \text{(Eq. 4)}$$

where k_{obs} = the observed rate constant, k_o = the rate constant at zero buffer concentration = the intercept of the lines with the y-axis, C = total buffer concentration, and $ka_1 = 8.9 \times 10^{-3}$ at 96°, and $\mu = 0.50$.

On this basis, $k_{H_3PO_4} = 3.64 \times 10^{-2}$ hours^{-1} mole^{-1} L.; $k_{H_2PO_4^-} = 4.13 \times 10^{-2}$ hours^{-1} mole^{-1} L.

Figure 3 shows the catalytic effect of phosphate buffers pH 6.00 to 7.50. The zero slopes of the lines representing the runs at pH 7.04 and pH 7.50, where more than 99% of the total ascorbic acid exists as the monohydrogen ascorbate ion, indicates that both

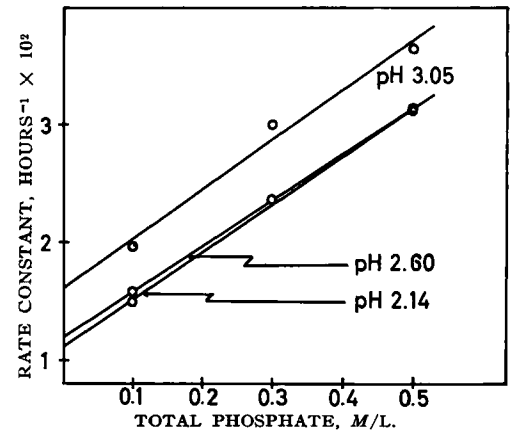


Fig. 2.—Plots showing the effect of phosphate concentration on the pseudo first-order rate constant of the anaerobic degradation of ascorbic acid at fixed pH values. All the runs were made at 96°C. and an ionic strength of 0.50.

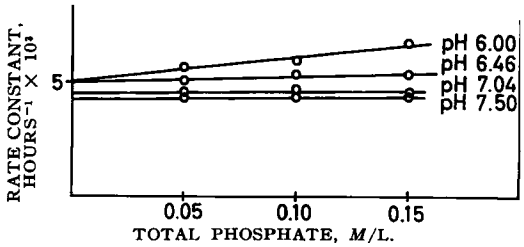


Fig. 3.—Plots showing the effect of phosphate concentration on the pseudo first-order rate constant of the anaerobic degradation of ascorbic acid at fixed pH values. All the runs were made at 96°C. and an ionic strength of 0.50.

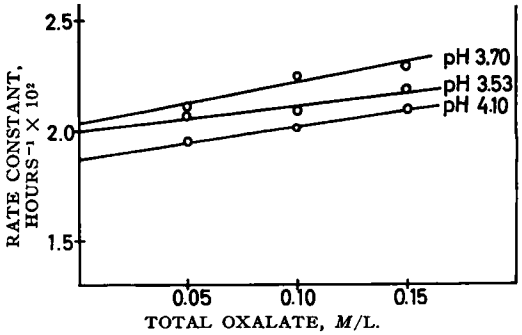


Fig. 4.—Plots showing the effect of oxalate concentration on the pseudo first-order rate constant of the anaerobic degradation of ascorbic acid at fixed pH values. All the runs were made at 96°C. and an ionic strength of 0.50.

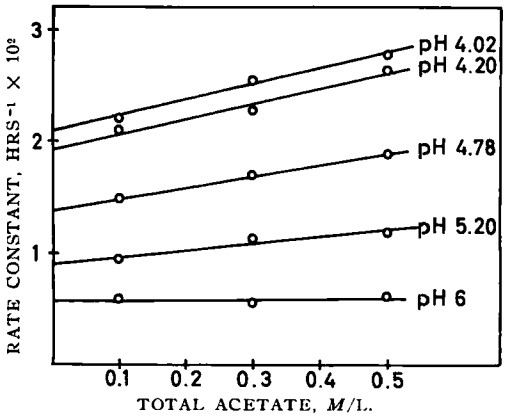


Fig. 5.—Plots showing the effect of acetate concentration on the pseudo first-order rate constant of the anaerobic degradation of ascorbic acid at fixed pH values. All the runs were made at 96°C. and an ionic strength of 0.50.

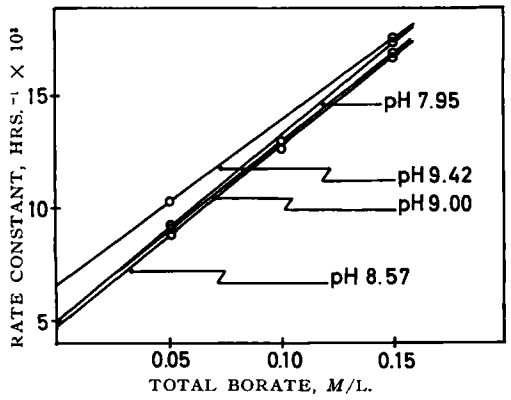


Fig. 6.—Plots showing the effect of borate concentration on the pseudo first-order rate constant of the anaerobic degradation of ascorbic acid at fixed pH values. All the runs were made at 96°C. and an ionic strength of 0.50.

$H_2PO_4^-$ and $HPO_4^=$ are noncatalytic with respect to this ion. The increasing slopes of the lines with decreasing pH may be attributed to the increase in the concentration of undissociated ascorbic acid with decreasing pH.

Oxalate buffers pH 3.7 to 4.1 and acetate buffers pH 4.02 to 6 have some catalytic effect (Figs. 4 and 5). Between pH 3.7 and 6 ascorbic acid will exist as undissociated acid and monohydrogen ascorbate ion. No attempt has been made to calculate the catalytic constants of the different species of these buffers with respect to different forms of ascorbic acid. The zero slope of the line on Fig. 5 representing the run at pH 6 seems to indicate, however, that CH_3COO^- is noncatalytic with respect to monohydrogen ascorbate ion. At pH 6, 99% of the total ascorbic acid will exist as the monohydrogen ascorbate ion, 95% of the total acetic acid will exist as the acetate ion.

The evaluation of the catalytic effect of borate buffers presents some problems because these buffers do not contain only undissociated H_3BO_3 and $H_2BO_3^-$ ions but also the ions BO_2^- and $B_4O_7^=$. The actual concentration of each of these ions cannot be readily determined. It is therefore quite difficult to make an accurate calculation of the ionic strength of the buffer solutions and to calculate the catalytic constant of each specie. The first of the difficulties mentioned may be handled by using the buffer in a relatively low concentration and adding a relatively great amount of a neutral salt. The ionic strength will then be determined mainly by the neutral salt. In our experiments the total buffer concentration never exceeded 0.15 *M* and sodium chloride was added to $\mu = 0.50$.

It is obvious from Fig. 6 that borate buffers have a pronounced apparent catalytic effect on the anaerobic degradation of ascorbic acid. At pH $\gtreqless$ 8.57, more than 98% of the total ascorbic acid will exist as the monohydrogen ascorbate ion. If we make the assumption that boric acid at pH $\gtreqless$ 8.57 will exist mainly as H_3BO_3 and $H_2BO_3^-$, it is possible to calculate the catalytic constants of these species with respect to the monohydrogen ascorbate ion.

We found $k_{H_3BO_3} = 8.03 \times 10^{-2}$ hours^{-1} mole^{-1} L.; $k_{H_2BO_3^-} = 7.81 \times 10^{-2}$ hours^{-1} mole^{-1} L.; pKa of boric acid was determined to be 8.59 at 96°; and $\mu = 0.50$.

It should be kept in mind that no attempt is made here to postulate the exact mode of catalysis. It is, of course, possible that an ester species may be involved with the borate system.

pH-Rate Profile of the Anaerobic Degradation of Ascorbic Acid.—The rate constants at zero buffer concentration can easily be picked from Figs. 2–6. The rate constants are shown in Fig. 7 as a function of pH. The rate constants given in Fig. 7 for pH<2 and pH>10 were found by making runs using perchloric acid solutions or sodium hydroxide solutions of known concentration. pH of the strongly acid solutions at 96° and $\mu = 0.5$ were calculated from

$$\text{pH} = -\log [H^+] + 0.08 \qquad \text{(Eq. 5)}$$

Equation 2 was used for the calculation of pH of the strongly basic solutions.

The pH rate profile (Fig. 7) suggests that the overall degradative rate represents a summation of a relatively large number of separate reactions. It is not possible from the experimental data to establish the correct reactions with certainty, but the following reactions would give a pH rate profile that appears to fit the experimental points well.

$$A + H^+ \xrightarrow{k_1} \text{products} \qquad \text{(Reaction 1)}$$

$$A \xrightarrow{k_2} \text{products} \qquad \text{(Reaction 2)}$$

$$A \cdot A^- \xrightarrow{k_3} \text{products} \qquad \text{(Reaction 3)}$$

$$A^- + A \xrightarrow{k_4} \text{products} \qquad \text{(Reaction 4)}$$

$$A^- \xrightarrow{k_5} \text{products} \qquad \text{(Reaction 5)}$$

$$A^= \xrightarrow{k_6} \text{products} \qquad \text{(Reaction 6)}$$

In these reactions, A = undissociated ascorbic acid, A^- = monohydrogen ascorbate ion, $A^=$ = ascorbate ion; and $A \cdot A^-$ = a complex of undissociated ascorbic acid and monohydrogen ascorbate ion.

The overall velocity is equal to the sum of the rates of all these reactions

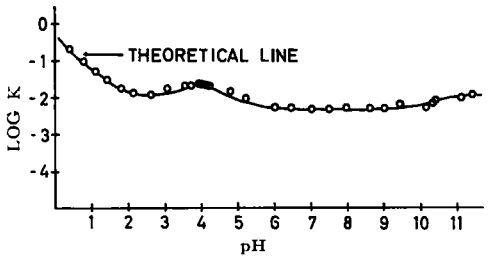


Fig. 7.—pH rate profile of the anaerobic degradation of ascorbic acid at 96°C. The circles represent the experimental results; the line corresponds to that expected theoretically from the six proposed reactions.

$$-\frac{d[A_T]}{dt} = k_1[A][H^+] + k_2[A] + k_3[A\cdot A^-] + k_4[A^-][A] + k_5[A^-] + k_6[A^=] \quad \text{(Eq. 6)}$$

$$[A_T] = [A] + [A^-] + [A^=] \quad \text{(Eq. 7)}$$

If we make the assumption that A^- will exist mainly bound as $A\cdot A^-$ at pH pKa_1, then $[A\cdot A^-]$ will be approximately equal to $[A^-]$.

At pH<pKa_1 $[A^=]$ will be negligible. This means that the following equation will be valid at pH<pKa

$$-\frac{d[A_T]}{dt} = k_1[A][H^+] + k_2[A] + k_3[A^-] + k_4[A^-][A] + k_5[A^-] \quad \text{(Eq. 8)}$$

Because of the overall first-order character of the reaction we have

$$-\frac{d[A_T]}{dt} = k\cdot[A_T] \quad \text{(Eq. 9)}$$

Combining Eqs. 7, 8, 9, and the equations

$$k_{a_1} = \frac{[H^+][A^-]}{[A]} \quad \text{(Eq. 10)}$$

$$k_{a_2} = \frac{[H^+][A^=]}{[A^-]} \quad \text{(Eq. 11)}$$

gives

$$k = \frac{k_1[H^+]^2 + k_2[H^+] + (k_3 + k_5)ka_1}{[H^+] + ka_1} + \frac{k_4ka_1[H^+]A_T}{([H^+] + ka_1)^2} \quad \text{(Eq. 12)}$$

Assuming A to exist mainly as $A\cdot A^-$ at pH>pKa_1, $[A\cdot A^-]$ will be approximately equal to $[A]$. At pH>pKa_1 $k_1[A][H^+]$ will be negligible compared to the other terms in Eq. 6 and we may write

$$-\frac{d[A_T]}{dt} = k_2[A] + k_3[A] + k_4[A^-][A] + k_5[A^-] + k_6[A^=] \quad \text{(Eq. 13)}$$

Combining Eqs. 7, 9, 10, 11, and 13 gives

$$k = \frac{(k_2 + k_3)[H^+]^2 + k_5ka_1[H^+] + k_6ka_1ka_2}{[H^+]^2 + ka_1[H^+] + ka_1ka_2} + \frac{k_4ka_1[H^+]^3A_T}{([H^+]^2 + ka_1[H^+] + ka_1ka_2)^2} \quad \text{(Eq. 14)}$$

From the experimental results the following k values have been calculated: $k_1 = 3.80 \times 10^{-1}$ hours^{-1}; $k_2 = 0.89 \times 10^{-2}$ hours^{-1}; $k_3 = 3.05 \times 10^{-2}$ hours^{-1}; $k_4 = 3.44 \times 10^{-1}$ hours^{-1} mole^{-1} L.; $k_5 = 5 \times 10^{-3}$ hours^{-1}; and $k_6 = 1.21 \times 10^{-2}$ hours^{-1}.

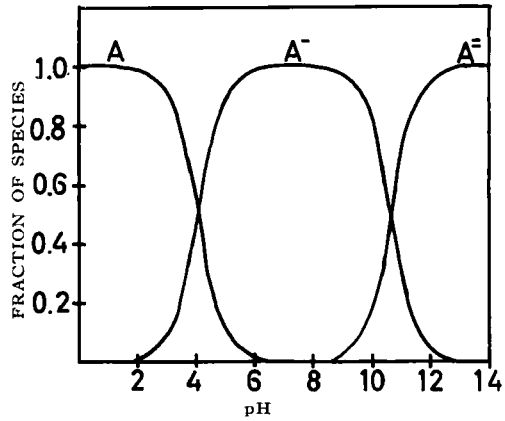


Fig. 8.—Fraction of ascorbic acid species in solution as functions of pH at 96°C. as calculated from the experimentally determined pKa_1 and pKa_2. A = Undissociated ascorbic acid, A^- = monohydrogen ascorbate ion, $A^=$ = ascorbate ion.

On the basis of the species profile shown in Fig. 8, the overall rate profile was calculated using Eq. 12 at pH<pKa_1 and Eq. 14 at pH>pKa_1; the result is shown as a solid line in Fig. 7.

Reaction 4 is suggested since it was found that the half life of the ascorbic acid degradation at pH 4 (= pKa_1 of ascorbic acid) was to some extent dependent on the total ascorbic acid concentration (Table III). No buffer was added in these experiments.

However, there is only a slight decrease in $t_{1/2}$ with increasing ascorbic acid concentration. This means that the apparent dominating reaction or reactions at pH 4 must be first order. Straight lines were obtained in each experiment by plotting the logarithm of residual ascorbic acid concentration against time.

Reaction 4 has nearly no influence on the shape of the calculated profile. At pH 4 where the influence is greatest, the calculated log k value is -1.65 with $k_4 = 3.44 \times 10^{-1}$, and -1.66 with $k_4 = 0$.

The relatively good agreement of the experimental data and the theoretical profile does not of course prove that the proposed Reactions 1–6 are the correct ones. Other reactions could lead to the same observed experimental dependencies.

At pH 6–9 the following three reactions would lead to the same log k/pH dependency

$$A^- \rightarrow \text{products}$$
$$A^= + H^+ \rightarrow \text{products}$$
$$A + OH^- \rightarrow \text{products}$$

The lack of primary salt effect at pH 6.5 indicates that the dominating reaction cannot be $A^= + H^+ \rightarrow$ products, but one of the other two. We have proposed the reaction $A^- \rightarrow$ products, but the reaction $A + OH^-$ cannot be eliminated.

At pH 10 to 11.5 the following two reactions would lead to the same log k/pH relationship

$$A^- + OH^- \rightarrow \text{products}$$
$$A^= \rightarrow \text{products}$$

TABLE III.—HALF-LIFE OF ANAEROBIC ASCORBIC ACID DEGRADATION AT 96°C. AND pH 4 AND DIFFERENT ASCORBIC ACID CONCENTRATIONS

Total Ascorbic Acid Concn., M/L.	$t_{1/2}$, hrs.
0.01	30.3
0.02	29.3
0.03	28.3
0.04	26.8
0.05	25.8

The lack of primary salt effect at pH 10.2 indicates that the dominating reaction cannot be $A^- + OH^- \rightarrow$ products but $A^- \rightarrow$ products.

Attempts to Prove the Existence of an Ascorbic Acid–Monohydrogen Ascorbate Complex.—Reaction 3 presupposes the existence of a complex between ascorbic acid and the monohydrogen ascorbate ion.

It is well known that organic acids are able to form complexes with their anions. Complex formation has been reported between mandelic acid and metal mandelate (7), salicylic acid and sodium salicylate (8), hippuric acid and potassium hippurate (8), succinic acid and potassium succinate (9), benzoic acid and sodium (10), and ammonium benzoate (11), *p*-hydroxybenzoic acid, dihydroxy benzoic acids, phenylacetic acid, adipic acid, saccharin, barbituric acid, barbital, phenobarbital, and their sodium salts (12).

The possible complex formation between ascorbic acid and monohydrogen ascorbate has been investigated by the authors by determination of freezing point depression, boiling point elevation, and solubility studies.

By calculation of the freezing point depression Δt, the following equation was used

$$\Delta t = p - q - 0.0125(p-q)(q-a)$$

where p = the freezing point of water, q = the freezing point of the solution, and a = the temperature to which the solution had been supercooled. Δt for a 0.5 M ascorbic acid solution was 0.943°. Δt for a 0.5 M ascorbic acid solution to which NaOH had been added to pH 4 was 1.301°.

For a 0.05 M ascorbic acid solution the corresponding values were 0.093 and 0.128°.

The boiling point elevation was determined according to Landsberger. There is by this method a condensation of vapor in the solution which is being studied. In our experiments we always used the same amount of a 0.5 M ascorbic acid solution and tried to get nearly the same amount of vapor condensed in each run. The final ascorbic acid concentration was therefore nearly the same in all experiments. Expressed as molality, it was 0.45. The average boiling point elevation Δt of the ascorbic acid solution with the final molality 0.45 was 0.19°. A solution of the same final molality but with NaOH added to pH 4 gave an average boiling point elevation $\Delta t = 0.27°$.

If the addition of sodium hydroxide to the ascorbic acid solutions (to pH 4) had caused no complex formation and if the influence of the ionic interactions in the solutions with pH 4 is neglected, the change in freezing point depression and boiling point elevation would have been 50%. The changes found were 38% for freezing point depression and 42% for the boiling point elevation. Taking into account the influence of the electrostatic attractions between the oppositely charged ions in the solutions with pH 4, our results show either that there is none or there is only a negligible complex formation between ascorbic acid and monohydrogen ascorbate, or the intermolecular forces in the complex are so weak that the complex formation cannot be detected by determination of freezing point depression or boiling point elevation.

Solubility studies were conducted according to the method of Higuchi and Lach (13). Five grams of ascorbic acid was added to 5 ml. of sodium monohydrogen ascorbate solutions of varying concentrations (0–3 M). The mixtures were placed in stoppered glass vials and rotated in a water bath at 30° ±0.1° overnight. An aliquot part of the clear solution was pipeted off and the ascorbic acid concentration determined by titration with 1 N sodium hydroxide.

Accurate results were impossible to obtain because of the high viscosity of the solutions. There was, however, a slight increase in the solubility of ascorbic acid with increasing salt concentration, thus indicating some complex formation. In our opinion it is difficult to draw definite conclusions from solubility studies in these very concentrated solutions and more so since an inseparable salting out effect cannot be excluded.

Our experiments do not prove the existence of a complex between ascorbic acid and monohydrogen ascorbate. On the other hand, they do not exclude this possibility. From a kinetical point of view a reaction that presupposes the existence of an ascorbic acid/ascorbate complex can explain the observed first-order character and the maximum in the pH rate profile at pH = pK_{a_1} of ascorbic acid.

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Catechol Monoglyceryl Ether Carbamates

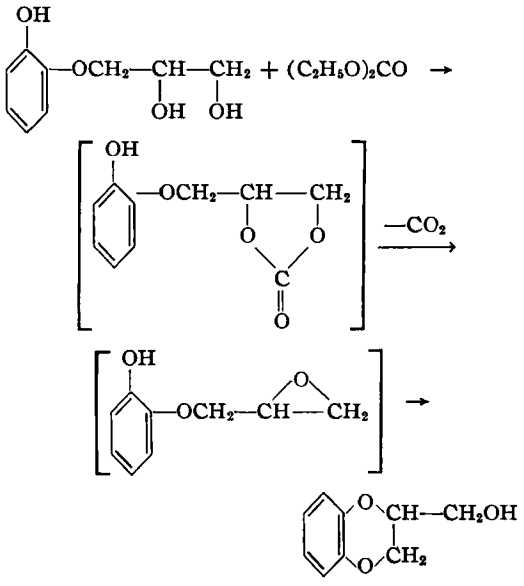
By J. SWIDINSKY, J. KERVENSKI, and B. B. BROWN

A series of carbamates and N-methylcarbamates derived from 3-(*o*-hydroxyphenoxy)-1,2-propanediol was prepared and screened for muscle relaxant activity. An ester interchange between the above diol and diethyl carbonate failed to give 4-(*o*-hydroxyphenoxymethyl)-1,3-dioxolone-2; 2-hydroxymethyl-1,4-benzodioxane was obtained instead. Prior benzylation of the phenolic hydroxyl resulted in the normal formation of the dioxolone. None of the compounds possessed muscle relaxant activity.

THE SUCCESS of 3-(*o*-methoxyphenoxy)-2-hydroxy-1-propyl carbamate, methocarbamol[1] as a muscle relaxant prompted evaluation of similar activity in carbamates derived from the monoglycerol ether of catechol, 3-(*o*-hydroxyphenoxy)-1,2-propanediol (1).

DISCUSSION

The monocarbamates were prepared by the reaction of ammonia and methylamine with 4-(*p*-benzyloxyphenoxymethyl)-1,3-dioxolone-2, followed by debenzylation with hydrogen over palladium-on-charcoal. The blocking of the phenolic hydroxyl group was required when it was discovered that 3-(*o*-hydroxyphenoxy)-1,2-propanediol would not form the corresponding cyclic carbonate, 4-(*o*-hydroxyphenoxymethyl)-1,3-dioxolone-2, upon ester interchange with diethyl carbonate. Instead 2-hydroxymethyl-1,3-benzodioxane was formed. It may be that the initially formed dioxolone decomposed under the conditions of the experiment to give the 1,2-epoxide which then cyclized to the dioxane by reaction with the phenolic hydroxyl



The activation of pyrolysis of cyclic carbonates by hydroxyl groups has been reported by D. B. Pattison (2). For example, he found that a mixture of 2,2-diethylpropanediol and its cyclic carbonate could be distilled at 220° at slightly reduced pressure, whereas the cyclic carbonate of trimethylolpropane decomposed rapidly at 180–200° to give 3-ethyl-3-oxetane methanol. It may well be that a phenolic group may exert a still more powerful influence on the elimination of CO_2 since the internal temperature during the experiment did not exceed 140°.

3-(*o*-Benzyloxyphenoxy)-1,2-propanediol (Table I, No. 1) was prepared by the reaction of 3-(*o*-hydroxyphenoxy)-1,2-propanediol with benzyl chloride in the presence of aqueous sodium hydroxide.

Compound 1 was heated with an excess of diethyl carbonate in the presence of sodium methylate to form 4-(*o*-benzyloxyphenoxymethyl)-1,3-dioxolone-2 (Table I, No. 2).

Compound 2 reacted readily with ammonia and methylamine in isopropyl alcohol solution to give in each case a mixture of two isomeric monocarbamates. These were separated by fractional crystallization into the primary and secondary monocarbamates, Table I, Nos. 3–6. The higher melting member of the pair was tentatively assigned the secondary carbamate structure (3).

Each of these monocarbamates was then debenzylated by hydrogenolysis over palladium-on-charcoal to the corresponding 3-(*o*-hydroxyphenoxy)-1(2)-2(1)-propyl carbamates (N-methylcarbamates) Table I, Nos. 7–10

3-(*o*-N-methylcarbamoxyphenoxy)-1,2-bis-N-methylcarbamoxypropane, Table I, No. 11, was prepared by the reaction of 3-(*o*-hydroxyphenoxy)-1,2-propanediol with excess methyl isocyanate.

The reaction of Compound 1 with methyl isocyanate yielded 3-(*o*-benzoyloxyphenoxy)-1,2-bis-N-methylcarbamoxypropane, Table I, No. 12. Debenzylation of Compound 12 gave 3-(*o*-hydroxyphenoxy)-1,2-bis-N-methylcarbamoxypropane, Table I, No. 13.

EXPERIMENTAL

Reaction of 3-(*o*-Hydroxyphenoxy)-1,2-propanediol with Diethyl Carbonate.—A mixture of 18.4 Gm. (0.1 mole) of 3-(*o*-hydroxyphenoxy)-1,2-propanediol, 35.4 Gm. (0.3 mole) of diethyl carbonate, and 0.5 Gm. of sodium methylate was heated until the distillation of ethyl alcohol ceased. A 1-Gm. quantity of ammonium chloride was added to destroy the sodium methylate. The excess diethyl carbonate was removed at reduced pressure and a maximum internal temperature of 140°. The resi-

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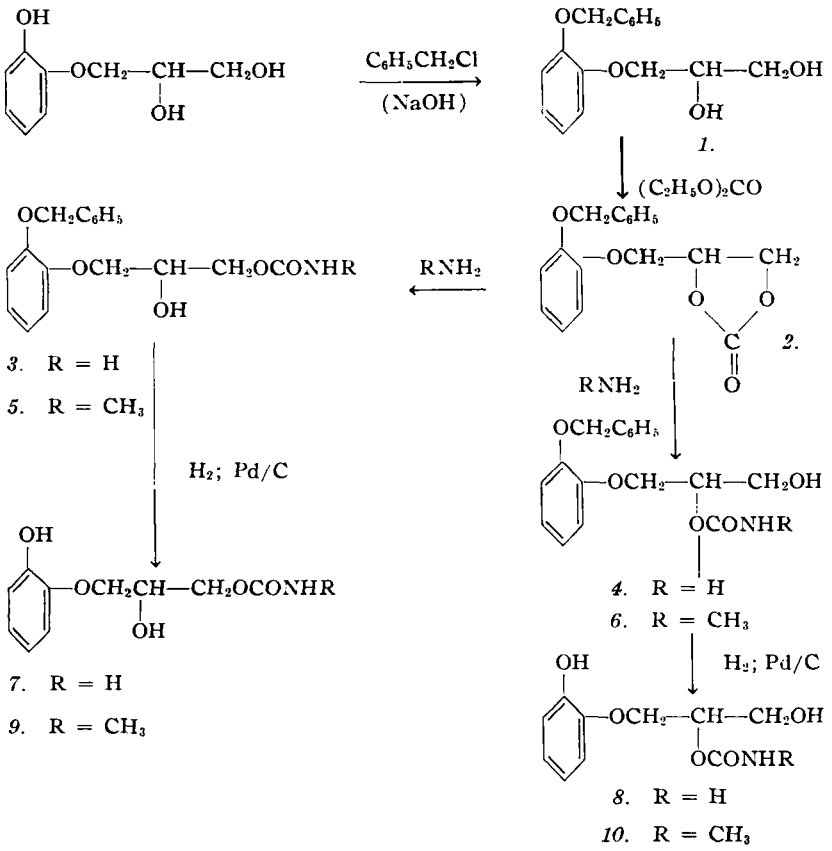
[1] Marketed as Robaxin by A. H. Robins Co., Inc., Richmond, Va.



due was extracted with boiling cyclohexane to give on cooling 7.9 Gm. of a solid, m.p. 83–86°. Recrystallized from ethyl acetate, it melted at 89–90°. A large depression in melting point was found on admixing with starting material (m.p. 90°). A test for a phenolic group with $FeCl_3$ was negative, and the infrared absorption spectrum showed no maximum for a carbonyl group. The IR absorption spectrum, however, was found to be identical with that of 2-hydroxymethyl-1,4-benzodioxane, which was prepared according to Grün (4) from epichlorohydrin and catechol, m.p. 89–90°. There was no depression in melting point on mixing the two compounds.

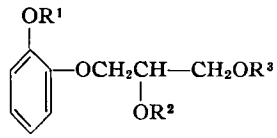
3 - (*o* - Benzyloxyphenoxy) - 1,2 - propanediol.—A mixture of 92.1 Gm. (0.5 mole) of 3-(*o*-hydroxyphenoxy)-1,2-propanediol, 62.5 Gm. (0.5 mole) of benzyl chloride and a solution of 24 Gm. (0.6 mole) of sodium hydroxide in 500 ml. water was heated to reflux with stirring for 2 hours. The mixture was cooled and the product extracted with ether. The ether was removed and the residue vacuum distilled to yield 82.1 Gm. (59.8%) of 3-(*o*-benzyloxyphenoxy)-1,2-propanediol-1, b.p. 200–218° at 0.4 mm. The product solidified on standing; the melting point was 81.0 to 82.5° after recrystallization from carbon tetrachloride. The crude distilled material was found to be suitable for subsequent reactions.

4 - (*o* - Benzyloxyphenoxymethyl) - 1,3 - dioxolone-2.—A mixture of 27.4 Gm. (0.1 mole) of 3-(*o*-benzyloxyphenoxy)-1,2-propanediol, 35.4 Gm. (0.3 mole) of diethyl carbonate, and 0.3 Gm. of sodium methylate was heated until ethyl alcohol distillation ceased (maximum internal temperature, 135°). A 0.5-Gm. quantity of ammonium chloride was added to destroy the sodium methylate, and excess diethyl carbonate was removed by vacuum distillation. The residue was dissolved in 400 ml. of hot isopropyl alcohol, treated with 0.5 Gm. of Darco G-60, filtered, and allowed to crystallize to yield 24 Gm. (80%) of 4 - (*o* - benzyloxyphenoxymethyl) - 1,3 - dioxolone-2, m.p. 74–75°.

3 - (*o* - Benzyloxyphenoxy) - 1(2) - hydroxy - 2(1)-propyl Carbamates and N-Methylcarbamates.—The general method for the preparation of these hydroxypropyl carbamates was as follows: ammonia or methylamine (1.2 moles) dissolved in isopropyl (or aqueous isopropyl) alcohol was reacted with compound 2, (1 mole). After standing at room temperature for 4–24 hours, the volatile material was distilled at reduced pressure, and the residue recrystallized from ethyl acetate or isopropyl alcohol to give the higher melting 1-hydroxy-2-carbamate (N-methylcarbamate). The residue from the mother liquor of this crystallization was then recrystallized from petroleum ether-benzene to give the lower melting isomer, the 2-hydroxy 1-carbamate (N-methylcarbamate). The yields of both isomers are based on the amount of Compound 2.

3 - (*o* - Hydroxyphenoxy) - 1(2) - hydroxy - 2(1)-propyl Carbamates and N-Methylcarbamates.—The general method for the removal of the benzyl group from the above benzyloxy carbamates was to shake an isopropyl alcohol solution of the benzyl ether with hydrogen at 2–5 p.s i. over 15% palladium-on-charcoal at 25–50° (the reaction mixture was heated if the hydrogen absorption was sluggish

TABLE I.—COMPOUNDS PREPARED



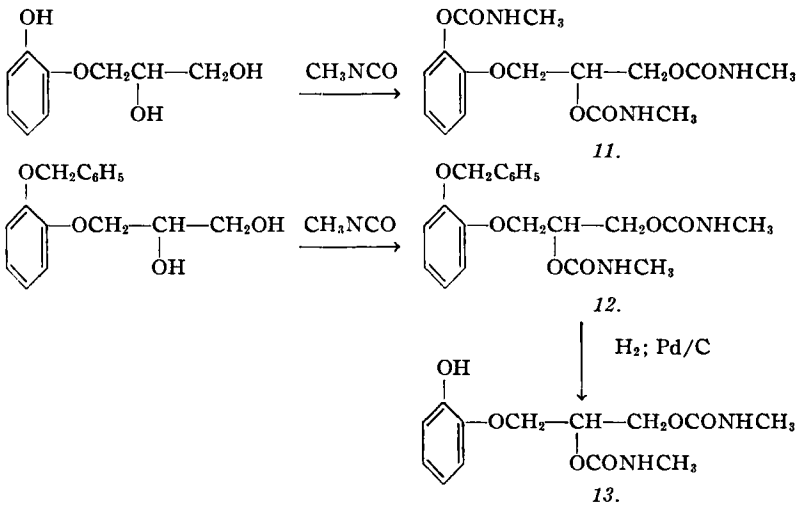
No.	R¹	R²	R³	M.p., °C.	Yield, %	Formula	Anal. Calcd. % C	Calcd. % H	Found % C	Found % H
1	$CH_2C_6H_5$	H	H	81–82.5	59.8	$C_{16}H_{18}O_4$	70.01	6.60	70.28	6.61
2	$CH_2C_6H_5$	—CO—		74–75	80.0	$C_{17}H_{16}O_5$	67.99	5.37	67.82	5.57
3	$CH_2C_6H_5$	H	$CONH_2$	73.6–74.8	24.7	$C_{17}H_{19}O_5N$	4.41% N		4.18% N	
4	$CH_2C_6H_5$	$CONH_2$	H	86.6–87.6	48.6	$C_{17}H_{19}O_5N$	4.41% N		4.21% N	
5	$CH_2C_6H_5$	H	$CONHCH_3$	53–55	58.2	$C_{18}H_{21}O_5N$	4.23% N		4.16 N	
6	$CH_2C_6H_5$	$CONHCH_3$	H	66.5–68	16.7	$C_{18}H_{21}O_5N$	4.23% N		4.12% N	
7	H	H	$CONH_2$	116–118	72.0	$C_{10}H_{13}O_5N$	6.17% N		6.02% N	
8	H	$CONH_2$	H	125–126	59.2	$C_{10}H_{13}O_5N$	6.17% N		6.06% N	
9[a]	H	H	$CONHCH_3$	76–78	45.7	$C_{11}H_{15}O_5N$	5.81% N		5.68% N	
10[a]	H	$CONHCH_3$	H	78–79.5	77.0	$C_{11}H_{15}O_5N$	5.81% N		5.71% N	
11	$CONHCH_3$	$CONHCH_3$	$CONHCH_3$	122–124	61.5	$C_{15}H_{21}O_7N_3$	11.85% N		11.34% N	
12	$CH_2C_6H_5$	$CONHCH_3$	$CONHCH_3$	122–124	54.7	$C_{20}H_{24}O_6N_2$	7.21% N		7.26% N	
13	H	$CONHCH_3$	$CONHCH_3$	55–57	54.3	$C_{13}H_{18}O_6N_2$	9.39% N		9.12% N	

[a] Mixed m.p. 73–76°.

or did not occur at room temperature). The reaction mixture was filtered from the catalyst, evaporated to dryness, and recrystallized from petroleum ether–benzene.

3-(*o*-N-Methylcarbamoxyphenoxy)-1,2-bis-N-methylcarbamoxypropane.—A benzene solution of 36.8 Gm. (0.2 mole) of 3-(*o*-hydroxyphenoxy)-1,2-propanediol was stirred with a large excess of methyl isocyanate and a few drops of pyridine as catalyst until the product crystallizing from the solution was no longer oily. Filtration yielded 43.7 Gm. (61.5%) of 3-(*o*-N-methylcarbamoxyphenoxy)-1,2-bis-N-methylcarbamoxypropane, Table I, No. 11; m.p. 122–124°.

3-(*o*-Benzyloxyphenoxy) and 3(*o*-Hydroxyphenoxy)-1,2-bis-N-Methylcarbamoxypropane.—A benzene solution of 19.2 Gm. (0.07 mole) of Compound 1 was reacted with an excess of methyl isocyanate and a few drops of pyridine. The product which precipitated was filtered to give 14.9 Gm. (54.7%) of 3-(*o*-benzyloxyphenoxy)-1,2-bis-N-methylcarbamoxypropane, Table I, No. 12, m.p. 122–124°. Compound 12, 11.6 Gm., was then debenzylated to 4.8 Gm. (54.3%) of 3-(*o*-hydroxyphenoxy) 1,2-bis-



N-methylcarbamoxypropane, Table I, No. 13, m.p. 55–57°, using the previously given procedure.

BIOLOGICAL RESULTS

All of the above carbamates except Compound 13, for which no suitable vehicle could be found, were screened for muscle relaxant activity in mice. A substance was said to possess muscle relaxant activity if a given dose caused the hind portion of the test animal to go limp while it was still able to walk on its front legs, dragging the back ones behind. All known muscle relaxants gave this test. Using this criterion, none of the above compounds showed significant muscle relaxant activity up to 1000 mg./Kg. However, the ability to delay the onset of pentylenetetrazol induced convulsions appeared to be general, particularly in Compounds 9 and 10.

No deaths to mice resulted from oral intubation of doses up to 1 Gm. per kilogram. Ataxia, paralysis, convulsions, and decreased respiration were notably absent in all tests at these levels.

REFERENCES

(1) Meltzer, R. I., and Doczi, J., *J. Am. Chem. Soc.*, **72**, 4986(1950); and Ueda, T., Toyoshima, S., Takahashi, K., Muraoka, M., Koibuchi, H., and Seto, Y., *Chem. Pharm. Bull. Tokyo*, **8**, 921(1960).
(2) Pattison, D. B., *J. Am. Chem. Soc.*, **79**, 3455(1957).
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(4) Grün, A., U. S. pat. 2,366,102(1944).

Toxicity of Plastics Used in Medical Practice I

Investigation of Tissue Response in Animals by Certain Unit Packaged Polyvinyl Chloride Administration Devices

By W. H. LAWRENCE†, J. L. MITCHELL‡, W. L. GUESS, and J. AUTIAN

In the past workers in our laboratory and other investigators have noted that polyvinyl chloride tubings used in medical practice, as administration or collection devices, will release one or more constituents to several types of solvent systems used in pharmacy. Since a great many formulations may be employed in manufacturing these plastic tubings, it was thought that a toxicity study might reveal if one or more of the currently used plastic administration devices might contain an ingredient which could produce a tissue response when implanted in animals. The results of the study revealed that under the experimental conditions used in this study a number of the tubings will produce tissue response while others will not.

For the past number of years, workers in our laboratory have attempted to focus attention on certain problems which might develop in the improper use of plastics, while at the same time encouraging research to develop products which would give the advantages of plastics without introducing potential hazards (1–5). Several approaches in research to the plastic problem have since been undertaken by the laboratory from an academic viewpoint and as a public health service. The work reported in this paper is the first of a series devoted to the exploration of the acute and toxic properties of plastics and the various ingredients which might be incorporated within the polymer to achieve a desired plastic which may be used in medical practice. Specifically, this paper will be devoted to ascertaining if certain plastic tubings (primarily of the polyvinyl chloride type) which are parts of administration devices might contain an ingredient or ingredients which could be considered toxic if released into animal tissue.

EXPERIMENTAL

Selection of Samples for Investigation.—Various types of administration devices having a polyvinyl chloride tubing as a component were obtained in their original package. A number of these packages indicated that the contents were sterile and nonpyrogenic. Each sample was assigned a code number with the manufacturer's name designated by a specific letter. Forty-eight different samples of administration devices from 17 manufacturers or distributors were employed in the investigation (see Table I). Several polyethylene tubings and one unidentified tubing used in a hospital were also included in the total number of samples.

Implantation Studies.[1]—In all the studies reported here only the tubings were evaluated, the other portions of the administration devices being stored for

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TABLE I.—INTRAMUSCULAR IMPLANTATION OF PLASTIC SAMPLES IN RABBITS FOR A PERIOD OF 7 DAYS[a]

Company (in code)	Sample (in code)	Result[b]	Remarks[c]
A	X-1	−	S. P.
B	X-19	+	S. P.
B	X-24	−	S. P.
B	X-25	+	S. P.
B	X-41	+	S. P.
B	X-44	−	S. P.
B	X-45	+	S. P.
B	X-49	+	S. P.
B	X-67	+	S. P.
B	X-74	+	S. P.
C	X-2	−	C. R.
C	X-17	+	C. R.
C	X-39	−	S. P. (B. T.)
C	X-54	+	S. (A. T.)
D	X-3	−	S. P.
D	X-50	−	S. P.
E	X-4	+	S. P.
E	X-48	+	S. P.
F	X-5	−	S. P.
F	X-69	−	S. P.
F	X-70	−	S. P.
F	X-71	−	S. P.
F	X-72	−	S. P.
F	X-73	−	S. P.
G	X-0	−	In roll
G	X-6	+	In roll
H	X-7	−	From opened package
H	X-66	−	S.
I	X-8	+	From opened package
I	X-61	+	S.
J	X-18	+	C. R.
J	X-21	+	S.
J	X-36	+	C. R.
J	X-37	+	S. (B. T.)
J	X-53	+	C. R.
J	X-63	+	C. R.
J	X-64	+	S.
J	X-65	+	S. (B. T.)
K	X-20	−	S. P. (S. T.)
L	X-23	−	S. (A. T.) polyethylene
L	X-52	−	S. (A. T.) polyethylene
L	X-68	−	(A. T.) polyethylene
M	X-40	+	No information concerning quality on package
N	X-47	−	S. P.
O	X-35	+	S. P.
P	X-60	−	S.
Q	X-43	−	S. P.
Q	X-55	+	Supplied to laboratory for evaluation

[a] If not designated, all tubings are of the vinyl type and are unit-packaged ready for use. [b] −, no reaction; + tissue reactions. [c] Information on package: S—sterile; P—nonpyrogenic; C. R.—clean, ready to use; (B. T.)—biologically tested; (A. T.)—animal tested; (S. T.)—safety tested.

future investigation. Sections of each sample were taken and cut into strips measuring approximately 1 mm. × 1.5 cm. and were implanted into rabbits in the following manner. For each sample two healthy, female, albino rabbits (1.6 to 2.0 Kg.) were used. Prior to an implantation, the rabbit's back

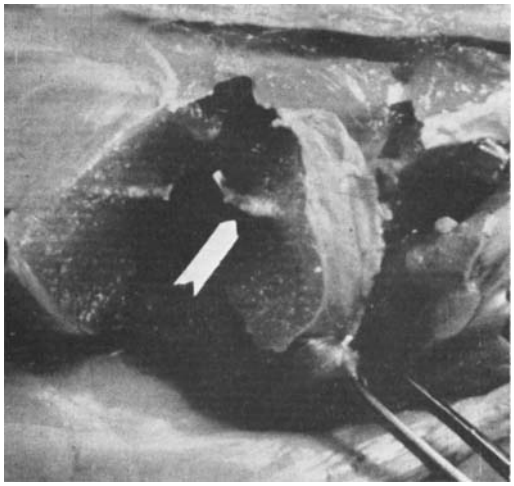

Fig. 1.—Photograph of an implantation site in rabbit muscle showing a sample of tubing X-74 (in center) after 1 week of implant. White-like zone around sample indicates tissue response.

was clipped and the excess hair removed by careful sponging (50% water-alcohol) with a lint-free towel. The rabbits were then anesthetized by an injection into the marginal ear vein of 0.6 ml./Kg. of a solution of pentobarbital sodium (50 mg./ml.) and stretched out on a table. A strip of the plastic was placed into the bevelled point of a 15 G needle having a length of 1.5 in., and the needle was introduced into the paravertebral muscle. Four strips of each sample were introduced (approximately 1 in. apart) into each animal. Two strips of a previously studied polyvinyl chloride tubing were also implanted in an identical manner as controls in the same rabbit.[2] The introduction of these plastic strips was done in a "clean" manner but not under aseptic conditions. Thiomersal solution was applied to all the sites of injection.

After implantation, each rabbit was returned to his cage and observed for a period of 1 week, at which time the animal was sacrificed and the paravertebral muscle exposed. Each implant site was isolated and examined macroscopically for tissue damage compared to the control samples. Tissue reaction or toxic reaction was indicated if an opaque or white-like zone was seen around the implant. Often the "toxic" strip showed an encapsulation which extended into the tissue for several mm. All the controls produced no apparent reaction other than the accepted mild trauma from the introduction of a foreign body. Any strip showing the tissue manifestation described above was regarded as toxic and was so recorded. If a question arose as to the accuracy of the observation, one or two more rabbits were included in the test. Invariably, if one strip showed a toxic effect, the other three strips showed exactly the same picture. The results of this 7-day study are included in Table I. Figure 1 shows a photograph of a section of muscle tissue (in rabbit) with an implanted strip (X-74). A white-like zone around the strip indicates the toxic response. Figure 2 is a subcutaneous implant (in rabbit) of sample

[2] Previous history of this sample has indicated that it is nonreactive to tissue.

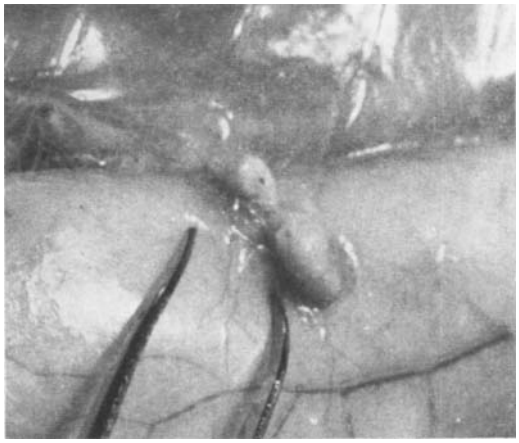

Fig. 2.—Photograph of an implantation site (subcutaneous) in rabbits of sample X-55 showing the toxic response (capsule formation around tubing).

X-55 and clearly demonstrates the formation of a capsule around the implant.

It will be noted that 25 of the 48 samples produced a toxic response under the conditions of the experiment.

Further Implantation Studies of Samples X-37 and X-74 in Rabbits.—It seemed highly desirable to investigate several of the toxic tubings in more detail, and for this reason samples X-37 and X-74 were selected, since adequate quantities of each were on hand with all the packages making up each sample having the same control number. It was felt that the samples should be of the same lot number throughout the investigations because it is known that at times a company may change its formulation for a tubing without revealing that this has been done.

In one series of experiments, several groups of rabbits were implanted with X-37 and X-74 by the intramuscular route as described. Both samples were also implanted by the subcutaneous route in several groups of rabbits. All animals were observed for a period of 28 days, a representative animal from each group being sacrificed at the end of each week for evaluation of toxicity.

Table II.—Reaction Induced in Rabbit Tissue by Samples X-37 and X-74 (Intramuscular Implantation)

Sample	Days 7	14	21	28
X-37[a]	*e*	*e*	*e*	*e*
X-74[a]	*d*	*b*	*b*	*b*
Control	*b*	*b*	*b*	*b*

[a] Four strips of each sample were used in each animal. Two control strips were also employed in the same animal. [b] Nonreactive. [c] Slightly reactive. [d] Moderately reactive. [e] Very reactive.

Table III.—Reaction Induced in Rabbit Tissue by Samples X-37 and X-74 (Subcutaneous Implantation)

Sample	Days 6	14	21	28
X-37[a]	*f*	*f*	*e*	*e*
X-74[a]	*e*	*c*	*c*	*d*
Control	*b*	*b*	*b*	*b*

[a] Four strips of each sample were used in each animal. Two control strips were also employed in the same animal. [b] Nonreactive. [c] Questionable. [d] Slightly reactive. [e] Moderately reactive. [f] Very reactive.

Table IV.—Reaction Induced in Rat Tissue by Samples X-37, X-55, and X-74 (Implantation in Thigh Muscle)

Sample[a]	Days 7	14	21	28	35
X-37	*g*	*d*	*e*	*e*	*e*
X-55	*f*	*f*	*f*	*f*	*f*
X-74	*d*	*g*	*c*	*c*	*b*

[a] One strip was placed into right thigh of the animal, and a control strip placed into the left thigh. [b] Nonreactive. [c] Questionable. [d] Slightly reactive. [e] Moderately reactive. [f] Very reactive. [g] Sample lost.

Tables II and III show the results and reveal that while X-37 persists to induce a toxic response, X-74 reaches a critical time period after which the toxic response appears to regress to a point where the sites are identical to the control samples.

Surgical implants of both X-37 and X-74 were performed in the brain tissue of the rabbits (five to each sample), and these animals were observed over a period of time up to 1 month. Within a period of 3 to 7 days, several of the rabbits were sacrificed and the brain tissue examined. Both samples produced tissue alteration but X-37 to the greatest degree. Within a 10-day period, several of the rabbits were observed to have a swelling immediate to the implant. Gross examination of the swollen area revealed that the cause of the swelling was because of the accumulation of fluid. It was first thought that an infection had caused the accumulation of fluid, but bacteriological examination of both the tissue and fluid proved this assumption to be false. Daily observation of these rabbits indicated that no general

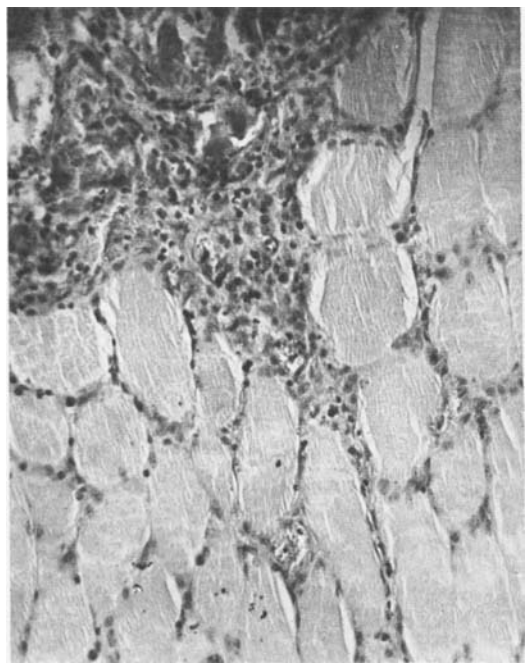

Fig. 3.—Photomicrograph of muscle tissue reaction in rabbit after implantation with sample X-37. Multinucleated giant cells and scattered polymorphonuclear leucocytes are in evidence (1 × 256).

pattern of behavior could be resolved to indicate the effect the implants had. Several of the rabbits died between the third and fourth week. Extensive brain damage could be seen in these rabbits after autopsy.

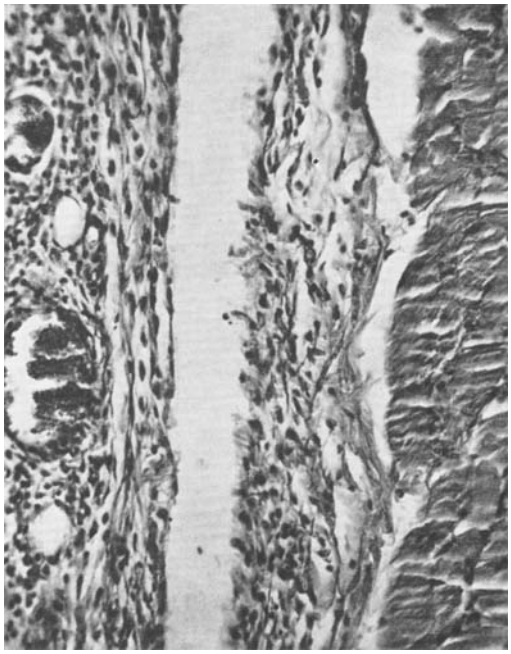

Fig. 4.—Photomicrograph of muscle tissue in rat after implantation with sample X-55. Severe damage to tissue may be noted by observing the formation of fiberous connective tissue (1 × 256).

Fig. 5.—Photomicrograph of muscle tissue in rabbit where a control plastic (nontoxic) was implanted. Mild inflammation may be noted at interface of plastic and tissue because of expected foreign body reaction (1 × 256).

Further Implantation Studies on Samples X-37, X-55, and X-74 in Rats and Mice.—To validate that the reactions seen in the rabbits were not specific to that animal, rats and mice were employed. The rats were of the Holtzman albino type, female, with an average weight of 300 Gm. Uniform samples of the three plastics were implanted by surgical procedure into a pocket formed between two layers of the thigh muscle in three groups of five rats. The implantation sites were closed by sutures. A nonreactive plastic strip (as employed with the rabbits) was implanted in the other leg of each rat as a control. Each week one rat from each group was sacrificed and the sites examined. Table IV summarizes the results.

Samples of the above tubings were surgically implanted into the nape of the neck of three groups of five mice (male, albino, average weight of 30 Gm.). The incisions were sutured, and the mice observed for a period of 1 week at which time they were sacrificed. Toxic reactions were noted for the three in the same order as the rats.

Histopathological Studies.—To rule out that the toxicity was not because of bacterial contamination, sterile transfer was made from a representative number of toxic implants and cultured on agar plates. The absence of growth after the 7-day and 14-day incubation period indicated that the tissue response was due to the plastic or a component in the plastic.

Representative implantation sites were carefully excised from the several animals employed in this study and placed into 10% formalin solution for histological examination. Sections were prepared and stained by the standard hematoxylin-eosin method and examined by a pathologist to confirm the toxic response noted by the macroscopic evidence. Figure 3 is a photomicrograph of tissue reaction (muscle of rabbit) due to X-37. Multinucleated giant cells and scattered polymorphonuclear leucocytes are in evidence indicating the destructive effect of the implant. Severe tissue damage in rats by X-55 is shown in a photomicrograph in Fig. 4. Damage to the tissue has reached a point where fiberous connective tissue may be observed around sections of the plant. Figure 5 is included to show the effect of the control sample of polyvinyl chloride implanted in rabbit muscle. Slight foreign body reaction may be noted along the interface of the plastic and the tissue.

Extraction of Toxic Substance from Plastic.—Ten grams of each sample (X-37 and X-74) were extracted with 75 ml. of 95% ethyl alcohol in a soxhlet extractor for a period of 12 hours. Both samples were removed from the apparatus and rinsed with distilled water, after which they were dried with lint-free towel. Four strips of each sample were implanted by the intramuscular route, and, likewise, the same number of samples were implanted by the subcutaneous route into separate rabbits. These series of animals were sacrificed after 1 week and the sites of implants carefully examined for tissue alteration. None of these strips produced a reaction which differed from the control implants.

The alcoholic extracts were evaporated until a syrupy liquid remained. Approximately 2 to 3 Gm. of the total weight of the plastic sample was extracted by the alcohol. In general the physical ap-

pearance of the syrupy liquid resembled a plasticizer. Strips of nonreactive plastic were placed in intimate contact with the extracts of both samples for a period of 24 hours at which time they were removed and blotted with a lint-free towel. These strips were then intramuscularly implanted in rabbits and the sites examined after 1 week. Each of the strips produced a tissue response indicating that the toxic substance or substances were being released to the alcoholic solvent.

Chromatographic Separation of Components from Extracts.—Alcoholic extracts were prepared, as described before, from samples X-37 and X-74. The solvent was slowly evaporated in a steam bath until the odor of ethanol was no longer apparent. Each sample was then chromatographed on an F & M scientific model 500 gas chromatograph.[3] The operating conditions of the chromatograph were as follows: injection port, 275°; oven, 250°; detector, 250°; helium flow 50 ml./minute.

A 1-M. 1/2-in. aluminum tube was filled with 20% DC silicone grease on 60–80 ASTM mesh chromosorb W[4] and served as the chromatographic column.

Chromatograms of each sample revealed a number of components had been extracted from both of the samples X-37 and X-74. The representative chromatograms of X-37 and X-74 are shown in Figs. 6 and 7. An analysis of both chromatograms indicated that approximately 95 to 99% of the extract consisted of a heavy component or components which were later ascertained to be the plasticizers.

The light components (in Figs. 6 and 7 these would constitute the components resolved on the column in a 16-minute period) were captured by condensing them in a U-tube packed with glass wool and chilled with a dry ice–acetone bath. The heavy fraction or fractions were likewise captured after 16 minutes. Both the light and heavy components were then eluted from the tube with isopentane and the solvent evaporated. Two fractions were then isolated for each sample. These consisted of a fraction of light components and a fraction of heavy components.

Nonreactive plastic strips were kept in contact with each fraction for a period of 24 hours at which time they were intramuscularly implanted into the rabbits. Two strips from each fraction were used since the quantity of the fractions was very small. After 7 days, the animals were sacrificed and the sites examined. The strips containing the light components for both X-37 and X-74 caused a tissue response, while the strips soaked in the heavy components showed no tissue response. No response was seen for the fractions from the control sample. Results of this particular experiment indicated that one or more components in X-37 and X-74 were the causitive agents in eliciting tissue response and that no apparent response was induced by the plasticizers.

It was hoped that various components from the fraction designated as the "light component" could be isolated for implantation studies to pinpoint the substance or substances causing the tissue response; but, unfortunately, the supply of the same lot number of X-37 and X-74 was depleted. This phase of the investigation, therefore, had to be terminated.

[3] F & M Scientific Corporation, New Castle, Del.
[4] From F & M Scientific Corporation, New Castle, Del.

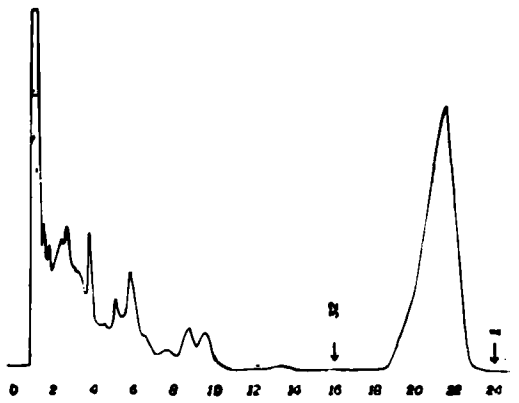

Fig. 6.—Gas chromatogram of extract from sample X-37 showing the number of components in the light fraction (up to 16 minutes) and the one component in the heavy fraction (plasticizer).

DISCUSSION

The results of the study reported in this paper indicate that there are now on the market tubings such as administration devices which contain one or more ingredients in microquantities which will migrate from the tubing and cause a toxic response in tissue when small samples are implanted. Forty-five of the 48 samples were of the vinyl type. Often for these particular tubings, approximately 40 to 60% of the total weight of the material will be the additives such as the stabilizers, antioxidants, colorants, and other components. The implantation studies revealed that 25 of these tubings caused a toxic response in a tissue. It is interesting to note that several manufacturers distributed a number of tubings which showed no tissue response, while other manufacturers produced tubings which showed a tissue response. One should also keep in mind that most of the tubings used in the study were packaged and ready for use. A number of these packages were labeled to indicate that safety tests had been performed on the device; others stated only that the package was sterile and nonpyrogenic.

In recent years several investigators have reported that certain polyvinyl chloride tubings contained one or more toxic ingredients (6). Generally, the causative agent was not the plasticizer but one of the other ingredients. This observation has been confirmed in the study reported in this paper, but it should not indicate that a particular plasticizer might not cause a toxic response under other experimental conditions.

Prior to and during the study, attempts were made to find the formulas used for the various tubings, but it soon became clear that the manufacturer would not, for obvious proprietary reasons, disclose his formula. Certain previous experiments also indicated to us that companies might change their formula for the tubing without indicating to the user that this in fact had been done.

Even though sterile conditions were not used in the implantation experiments, infections were ruled out as the causative agents in producing the toxic responses. If infection was indeed the causative agent, it would certainly have been noted with one or more of the control samples since these were implanted under identical experimental conditions. Confirma-

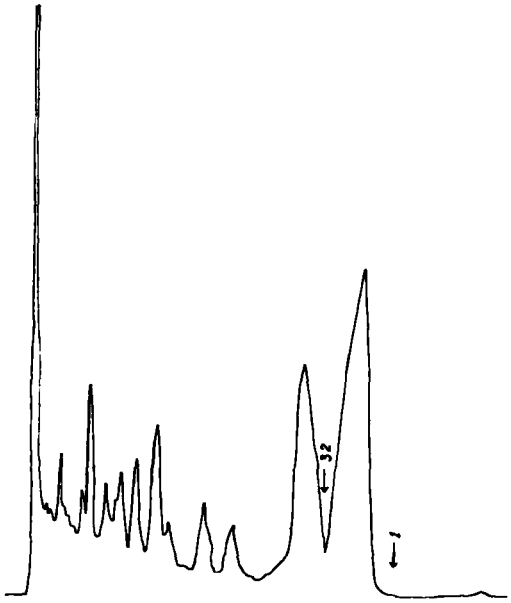

Fig. 7.—Gas chromatogram of extract from sample X-74 showing the number of components in the light fraction (up to 16 minutes) and the two components in the heavy fraction (plasticizers). Note: Same time scale as in Fig. 6.

tion of this fact was made by the bacteriological tests on the "toxic" tissue.

Further investigations with X-37 and X-74 revealed that the toxic ingredient was not because of the component making up the largest bulk of the ethanol extract—the plasticizer or plasticizers—but rather because of one of the number of components referred to as the "light fractions." There was no basis for assuming that the toxic ingredient was a contaminant picked up by the plastic during the manufacturing or packaging process since other tubings from the same manufacturer also showed a toxic effect. Certain investigators have concluded that toxic substances in polyvinyl chloride tubings are probably due to one or more organo-metallic compounds used as stabilizers (6, 7).

The implantation results for X-37, X-55, and X-74 were rather interesting since they did show that the degree of toxicity varied from sample to sample, X-55 eliciting the most severe response while X-74 the least. Sample X-74 lost its toxic activity with time, indicating that the animals were able to nullify or detoxify the offending agent in the plastic.

The implantation method for testing the toxicity of plastics was first developed by Brewer and Bryant (8), who found that certain disposable plastic items could cause a tissue response. This laboratory has since confirmed the value of the implantation technique because it can reveal in a very short period of time (from 3 to 7 days) that the particular plastic item has a component which may be considered undesirable. Cruickshank, *et al.* (9), has suggested the use of tissue cultures as a testing or screening method for plastics.

The question, of course, may now be raised that the tubings which are reported in this paper were never intended to be implanted either in animals or humans; consequently, for their intended use, they may be quite safe. Questions of this sort can best be answered by stating that good public health practice would seem to dictate that no plastic item should contain an ingredient which has a potential harmful ingredient that might leach into a solution to be administered to a patient. Furthermore, since there are tubings which have not shown toxic effects under the experimental conditions employed in this paper, it would seem prudent for manufacturers to re-examine their tubing formulations to remove those offending agents which may become potential sources of danger to patients.

SUMMARY

Forty-eight plastic administration devices were obtained from various sources. For the most part these devices were unit-packaged in sealed or closed packets or cartons. In general the tubings were of the vinyl type. Sections of the tubings from the various devices were investigated by implantation techniques into rabbits, rats, and mice. Morphological and histopathological examinations were used to detect toxic responses in the implanted tissue. The results revealed that 25 of the 48 samples used in the study produced a toxic response. Gas chromatographic techniques with implantation tests suggested that the toxic ingredient or ingredients in several of the tubings were a direct consequence of one or more of the additives, exclusive of the plasticizers.

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Interaction of Antihistamines with Hydrocolloids

By HORACE D. GRAHAM and YEDE MARIE BAKER†

Antihistamines of various types form highly insoluble complexes with carrageenan and other sulfated hydrocolloids. The carboxylic acid type hydrocolloids and the proteins, casein and gelatin, formed less insoluble complexes; the neutral polysaccharides and agar (supposedly sulfated) formed gels in the presence of the antihistamines. Pyrathiazine, chlorcyclizine, pyrilamine, and pyrrobutamine formed more insoluble complexes than thenyldiamine, methapyriline, benadryl, and thonzylamine when mixed with carrageenan. The interaction was accelerated by increased temperature up to 75°, was not appreciably affected by pH within the range of pH 2.75 to 6.55 and, at 45°, reaction times of 5–70 minutes produced no essential difference in the amount of benadryl hydrochloride bound per unit of carrageenan (Seakem type 5).

HYDROCOLLOIDS of various types are being added to pharmaceutical products with increasing frequency. Since many of these hydrocolloids are polyanionic, they can enter into strong interactions with cationic drugs such as many of the antihistamines and may affect drug insolubilization, appearance, stability, and prolongation of activity.

Kennon and Higuchi (1) have studied the interaction of benadryl and pyribenzamine hydrochlorides with sodium carboxymethylcellulose (CMC), one of the relatively weaker binders. These studies were undertaken to examine the interaction of a broad spectrum of hydrocolloids with several antihistamines, since any insoluble product formed through such interactions may be of value as sustained release form of the drugs, a topic which has been receiving much attention recently (2–5).

EXPERIMENTAL

Antihistamines.—Pyrrobutamine, pyrilamine maleate, WY 2149, benadryl, pyrathiazine, chlorcyclizine, phenergan, thenyldiamine, methapyrilene, thonzylamine, triprolidine, antistine, and pyribenzamine hydrochlorides were used as received from the various manufacturers and were made up in double distilled water.

The hydrocolloids, other reagents, and equipment and the experimental details have been previously described (5). Hypnean was prepared according to Smith and Montgomery (6).

Where precipitation did not occur, the equilibrium dialysis technique (1, 7) was employed and binding data were obtained using the technique of Hughes and Klotz (8).

The degree of dissociation of the polyelectrolytes and the significance of the Donnan effects were ascertained by the technique of Kennon and Higuchi (1).

Quantitative Determination of the Antihistamines.—For determining the amount of free antihistamine in the experimental systems, the color reactions of Osol and Sideri (9) and the ultraviolet absorption characteristics outlined by Kleckner and Osol (10) were exploited.

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RESULTS AND DISCUSSION

The sulfated and carboxylic acid type polysaccharides and the proteins formed insoluble complexes with all of the antihistamines tested, while the neutral polysaccharides and agar (supposedly sulfated) produced a gel rather than a precipitate. The antihistamines varied in their ability to precipitate the hydrocolloids and—if carrageenan (Seakem type 5) is taken as the representative hydrocolloid—the relative tendencies of benadryl, pyrrobutamine, pyrathiazine, chlorcyclizine, phenergan, pyrilamine, thenyldiamine, methapyriline, and thonzylamine to form precipitates with this hydrocolloid were calculated to be 1.0, 3.65, 3.54, 1.98, 2.54, 0.96, and 0.28, respectively. Table I and Fig. 1 show the maximum binding capacities of several hydrocolloids for many of the antihistamines used. The high binding capacity of carrageenan is clearly indicated. The maximum binding capacity of furcellaran is slightly less than one-half that of carrageenan, probably a reflection of the sulfate content of these two phytocolloids. CMC, sodium alginate, pectin, and quince seed mucilage differed only slightly in their maximum binding capacities for the antihistamines listed. Of the hydrocolloids listed, locust bean gum and gum guar exhibited the lowest binding capacities for the antihistamines.

Figures 2 and 3 show the extent of the Donnan effects on the binding of several of the antihistamines by CMC and carrageenan (Seakmen type 5). The degree of dissociation of CMC was found to be 61.6% and agrees well with the value of 60% reported by Kennon and Higuchi (1). Carrageenan (Seakmen type 5) was found to be 79.5% dissociated. Harwood (11) calculated the ionization of a 1.5% solution of this hydrocolloid to be 59% and, assuming a molecular weight of 1000, calculated the degree of ionization of a 0.01 *N* solution to 63.4% at 25°. The drastic deviations from the theoretical Donnan-only behavior, in all cases, suggest that the drug cations are bound by the hydrocolloid anions by other mechanism(s). This is substantiated by the gross observations where turbidity occurred when most of the antihistamines were mixed with the hydrocolloids and by the data (Table I) for the maximum binding capacities. When dilute systems of gum tragacanth and sodium alginate were similarly treated, corresponding great deviations from the theoretical curve were observed, again supporting the gross observations and the binding data. Since the antihistamines are cationic in nature, salt formation is probably the most pre-

TABLE I.—MAXIMUM BINDING CAPACITY OF HYDROCOLLOIDS FOR ANTIHISTAMINES, MICROMOLES OF ANTIHISTAMINE BOUND PER GRAM OF HYDROCOLLOID

	Antihistamine				
	Benadryl HCl	Pyrrobutamine	Pyrathiazine HCl	Phenergan HCl	Pyrilamine Maleate
Carrageenan	5,520	20,000	18,640	9,840	13,800
Furcellaran	3,000	9,800	8,987	5,675	6,800
CMC	820	2,680	2,365	1,300	2,020
Sodium alginate	960	2,600	2,300	1,656	2,600
Pectin	801	2,400	2,265	2,160	2,350
Gum tragacanth	662	1,987	1,740	1,060	1,232
Gum arabic	340	1,086	884	640	740
Quince seed mucilage	878	2,260	1,984	1,590	1,960
Locust bean gum	320	301	304	267	470
Gum guar	300	304	265	285	340

dominant reaction occurring between the polyanionic polysaccharides and the drugs, but hydrogen bonding and van der Waals forces probably also contribute to the total binding.

Figure 4 shows the effect of pH and electrolytes on the binding of benadryl hydrochloride by carrageenan (Seakem type 5). Determinations of the amount of benadryl hydrochloride bound by carrageenan (Seakem type 5) in the presence of several salts revealed that the order of interference may be summarized as: trivalent > divalent > monovalent. Iron and aluminum are particularly interferring and will actually precipitate the hydro-

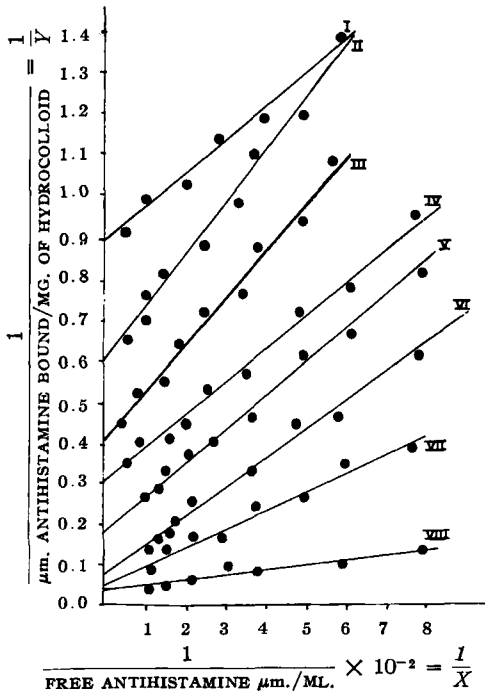

Fig. 1.—Plot of *1/y vs. 1/x* for limiting binding capacity of hydrocolloids for antihistamines. Data from studies at 27°C. in aqueous solutions. Key: I, pectin-benadryl hydrochloride; II, gum tragacanth-phenergan; III, sodium alginate—phenergan hydrochloride; IV, furcellaran—benadryl hydrochloride; V, carrageenan (Seakem type 5)—benadryl hydrochloride; VI, carrageenan (Seakem type 5)—pyrilamine maleate; VII, carrageenan (Seakem type 5)—pyrathiazine hydrochloride; VIII, carrageenan (Seakem type 6)—pyrrobutamine.

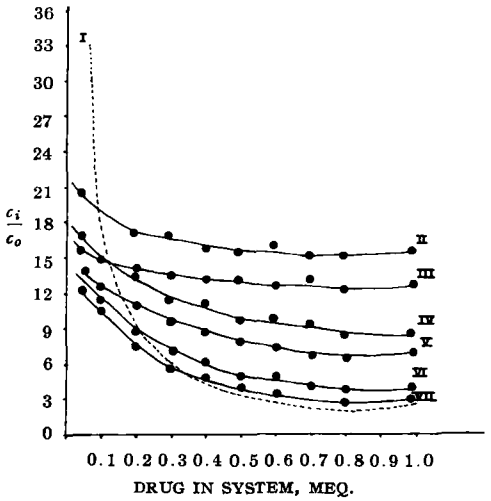

Fig. 2.—Illustration of the binding tendency of CMC toward antihistamines in aqueous solutions at 27°C. Key: I, Donnan; II, pyrrobutamine; III, pyrilamine; IV, pyrathiazine; V, phenergan; VI, benadryl; VII, pyribenzamine.

colloids. Reduced binding of the antihistamines by the hydrocolloids in the presence of electrolytes must be mainly because of the competition for the reactive sites on the antihistamine molecule.

The pH of the medium influences the interaction of the antihistamines with the hydrocolloids. In the alkaline range, the antihistamines will produce a turbidity in the absence of the hydrocolloids. Between pH 2.75 to 6.55 and, in distilled water, only slight differences in the amount of antihistamine bound per milligram of carrageenan (Seakem type 5) were observed. This constancy over such a wide range of pH would probably not hold for hydrocolloids such as CMC whose ionization and stability are severely affected at low pH levels of the medium. In studies on the effect of pH, hydrochloric acid was used to attain the desired acidity. If phosphate buffer is used, then the maximum strength (final molarity of the salt in the medium) must be 0.05 *M*. Beyond this, the amount of antihistamine bound per unit of hydrocolloid decreased, presumably because of the electrolyte effect (Fig. 4).

As the temperature of the interaction medium increased, the reaction between the hydrocolloid and the antihistamine increased, as gauged by the amount of antihistamine bound per milligram of

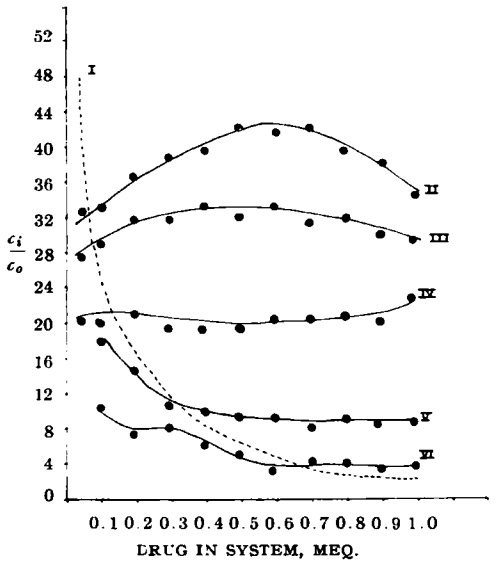

Fig. 3.—Illustration of the binding tendency of carrageenan (Seakem type 5) toward antihistamines in aqueous solutions at 27°C. Key: I, Donnan; II, pyrrobutamine; III, pyrathiazine; IV, pyrilamine; V, phenergan; VI, benadryl.

hydrocolloid. This held true up to 75°. Between 75–85°, there was no measurable difference in binding. Although 45° was not the optimum interaction temperature, it was selected to minimize the possibilities of hydrolysis if the reaction was carried out at higher temperatures.

At 45°, reaction times of 5–70 minutes produced no essential difference in the amount of benadryl hydrochloride bound per milligram of carrageenan (Seakem type 5).

Sustained or timed release of drugs from complexes has been receiving much attention recently (2, 4, 5); the interaction products of hydrocolloids and antihistamines may be of significance in this respect. Table II indicates the solubility of the benadryl-carrageenan and pyrilamine-carrageenan complexes in sodium chloride and hydrochloric acid. These data point to their promise as sustained-release forms of the drugs.

Antihistamines have a great number of pharmacological actions and, though *in vitro* studies may be inconclusive, they may throw some light on *in vivo* mechanisms. Judah (12), in his studies on the interaction of antihistamines *in vitro*, suggested that they prevented mitochondrial swelling by interactions with the mitochondrial membrane. Conceivably, substances closely related to some of the hydrocolloids used in these studies (*e.g.*, heparin, proteins, or chondroitin sulfate) could have been involved. Kobayashi (13) has discussed the binding of histamine by heparin with particular attention to the importance of the sulfate moiety of this compound in the binding process. Since the antihistamines are "histamine antagonists," the strong binding of the antihistamines by the sulfated hydrocolloids reflect the possible role of such types of compounds in the mechanism of action of the antihistamines. The role of proteins in the binding

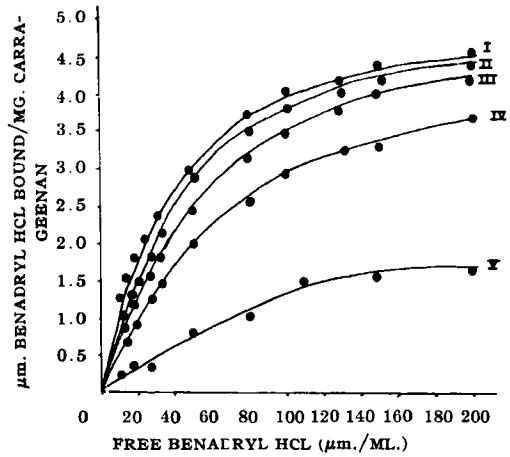

Fig. 4.—Effect of pH and electrolytes on the binding of benadryl hydrochloride by carrageenan (Seakem type 5) at 27°C. Key: I, distilled water; II, pH 2.75 to 6.55; III, sodium chloride (10^{-2} mole); IV, barium chloride (10^{-3} mole); V, ferric chloride (10^{-5} mole).

TABLE II.—SOLUBILITY OF BENADRYL- AND PYRILAMINE-CARRAGEENAN[a] COMPLEXES IN SODIUM CHLORIDE AND HYDROCHLORIC ACID AT 27°C.

	Benadryl Complex			Pyrilamine Complex		
	Bound antihistamine (%) released after					
	1 hr.	6 hr.	24 hr.	1 hr.	6 hr.	24 hr.
Sodium Chloride (Final Molarity)						
0.0	14.7	16.8	17.5	11.0	11.2	15.3
0.2	43.7	73.4	74.0	49.3	72.2	82.5
0.4	75.0	75.2	75.0	64.3	80.8	83.0
1.6	75.8	75.5	76.6	65.1	64.2	82.5
2.4	75.8	76.2	77.7	66.0	79.2	82.5
3.2	76.6	77.2	77.7	66.7	84.3	82.5
4.0	78.0	79.0	79.4	68.7	84.3	84.3
Hydrochloric Acid (Final Normality)						
0.00	14.7	15.8	17.5	10.2	10.5	15.0
0.01	29.5	57.6	68.4	58.0	58.0	71.0
0.10	63.2	66.8	71.7	62.0	63.0	84.0
0.20	72.2	72.4	72.2	67.0	67.3	84.0
0.50	72.0	72.2	71.7	75.0	71.0	81.0
1.00	71.8	72.0	70.0	75.0	75.0	89.0
2.00	72.0	72.4	70.0	74.0	75.0	89.0

[a] Seakem type.

of antihistamines is also of interest. Maietta (14, 15) has exploited such interactions in using the combined antigen-antihistaminic technique in shortening the treatment of hay fever.

These studies demonstrate that a wide variety of hydrocolloids and antihistamines form highly insoluble complexes which probably can serve as sustained-release forms of these drugs. Conceivably, soluble complexes which are probably formed in most of the systems studied also may be of pharmacological importance.

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Effectiveness of Antibacterial Agents Presently Employed in Ophthalmic Preparations as Preservatives Against *Pseudomonas aeruginosa*

By S. ROBERT KOHN†, LOUIS GERSHENFELD, and MARTIN BARR

Seven chemical substances or combinations of these substances presently employed as preservatives in ophthalmic solutions were studied to note their effectiveness as antibacterial agents against 13 different strains of *Pseudomonas aeruginosa*. New *in vitro* methods were devised. Among the latter were techniques to (*a*) differentiate between bacteriostatic and bactericidal activities and (*b*) determine the sterilizing time for each antibacterial agent. The methods presented here have several advantages over those previously employed and those now in use. An *in vivo* procedure was also employed in evaluating these chemical agents to note whether the findings were in agreement with the final results obtained in the *in vitro* studies. The following chemicals were examined: chlorobutanol, benzalkonium chloride, thimerosol, combinations of methyl and propylparaben, phenylmercuric nitrate, phenylethyl alcohol, and polymyxin B sulfate.

BECAUSE OF THE incidence and seriousness of *Pseudomonas aeruginosa* (*Ps. aeruginosa*) infections resulting from the use of contaminated ophthalmic solutions (1–11), various workers have critically investigated the antibacterial agents employed as preservatives in such preparations. The findings of these workers have been contradictory (12–18). The *in vitro* procedures employed in evaluating the effectiveness of the antibacterial agents have been challenged. The following observations are noted. There is need for methods which will determine the effectiveness of (*a*) antibacterial agents used as preservatives in ophthalmic solutions against *Ps. aeruginosa;* and (*b*) substances which are capable of inactivating or inhibiting the antibacterial action of the preservatives used.

The purpose of this paper is to report on studies which were performed in an effort to develop *in vitro* methods more effective than those employed at present and which will establish the efficiency of these antibacterial agents as preservatives in ophthalmic solutions against *Ps. aeruginosa.*

GENERAL CONSIDERATIONS

In devising methods for the evaluation of the effectiveness of antibacterial agents as preservatives in ophthalmic solutions, it is important to develop a technique which will determine the time required for such agents to produce sterility. Most methods which have been used to date did not always take this into consideration. Indeed, they usually measured only the bacteriostatic activity of preservatives.

Until recently, most workers employed a dilution technique to differentiate between the bactericidal and bacteriostatic activities of antibacterial agents. The basis for this procedure is the dilution of the

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of antihistamines is also of interest. Maietta (14, 15) has exploited such interactions in using the combined antigen-antihistaminic technique in shortening the treatment of hay fever.

These studies demonstrate that a wide variety of hydrocolloids and antihistamines form highly insoluble complexes which probably can serve as sustained-release forms of these drugs. Conceivably, soluble complexes which are probably formed in most of the systems studied also may be of pharmacological importance.

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Effectiveness of Antibacterial Agents Presently Employed in Ophthalmic Preparations as Preservatives Against *Pseudomonas aeruginosa*

By S. ROBERT KOHN†, LOUIS GERSHENFELD, and MARTIN BARR

Seven chemical substances or combinations of these substances presently employed as preservatives in ophthalmic solutions were studied to note their effectiveness as antibacterial agents against 13 different strains of *Pseudomonas aeruginosa*. New *in vitro* methods were devised. Among the latter were techniques to (*a*) differentiate between bacteriostatic and bactericidal activities and (*b*) determine the sterilizing time for each antibacterial agent. The methods presented here have several advantages over those previously employed and those now in use. An *in vivo* procedure was also employed in evaluating these chemical agents to note whether the findings were in agreement with the final results obtained in the *in vitro* studies. The following chemicals were examined: chlorobutanol, benzalkonium chloride, thimerosol, combinations of methyl and propylparaben, phenylmercuric nitrate, phenylethyl alcohol, and polymyxin B sulfate.

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preservative far below the concentration necessary to have a significant antibacterial effect. If growth of the bacteria occurred, after inoculation of this diluted solution into a subculture medium, the antibacterial agent was considered to possess bacteriostatic activity; if growth did not occur, then the antibacterial agent was reported as possessing bactericidal properties.

The above concept is not considered entirely valid at present. As Klarmann (19) has reported, in some of the dilution techniques, a quantity of the antibacterial agent may be transferred to the subculture medium in a concentration which will produce stasis, and some may become affixed to the cell walls or cells, initially producing stasis. Therefore, a bacteriostatic effect may be produced and misinterpreted for a bactericidal effect. Another disadvantage of the dilution technique is that living bacteria present may be diluted to an infinite concentration and may not grow in the subculture medium.

It is therefore apparent that an important factor in determining the antibacterial activity of preservatives in ophthalmic solutions is the complete inhibition or inactivation of the antibacterial agent in the evaluation procedure, a principle employed by Lawrence (16) and Riegelman, *et al.* (17).

Until recently, investigators had not attempted to correlate the results obtained using the *in vitro* procedures with an *in vivo* test. As pointed out by Riegelman, *et al.* (17), an *in vivo* test has several advantages and becomes an important consideration in procedures used for the evaluation of antibacterial agents employed as preservatives in ophthalmic solutions.

EXPERIMENTAL

The media employed in the experiments are listed in Table I.

General In Vitro Methods

To determine the effectiveness of presently employed antibacterial agents as preservatives for ophthalmic solutions against *Ps. aeruginosa*, four experimental *in vitro* procedures, each with a specific objective, were developed. These are described below.

Experiment I

Purpose.—As previously noted, it is important to distinguish between bacteriostatic and bactericidal activity in evaluating the effectiveness of chemical agents for use as preservatives in ophthalmic solutions. Various substances have been recommended as inactivating or neutralizing agents for the antibacterial activity of the chemicals employed. However, data concerning the effectiveness of inactivating agents are meager. Therefore, *Experiment I* was devised to determine and compare the effectiveness of various media for their ability to inhibit the activity of antibacterial agents against *Ps. aeruginosa*.

Preparation of the Inoculum.—Thirteen strains of *Ps. aeruginosa*, obtained from various sources, were identified by the usual methods including also the cytochrome oxidase test (20, 21), then maintained on agar slants. Two strains of *Ps. aeruginosa*, which were found more resistant to many of the antibacterial agents in preliminary studies were selected for use in this study. Prior to the day of the test, these strains were transferred for at least 3 consecutive days into 10 ml. of nutrient broth and incubated at 37° for 24 hours. On the day of the

TABLE I.—MEDIA EMPLOYED

Medium I (pH 6.8)		
Nutrient broth, dehydrated[a,f]		0.8 Gm.
Purified water	*q.s. ad.*	100 ml.
Medium II (pH 6.8)		
Glycerin[b]		4 Gm.
Medium I	*q.s. ad.*	100 ml.
Medium III (pH 6.8)		
Lecithin[c]		0.5 Gm.
Glycerin		4 Gm.
Medium I	*q.s. ad.*	100 ml.
Medium IV (pH 6.7)		
Tween 80[d]		3 Gm.
Medium I	*q.s. ad.*	100 ml.
Medium V (pH 6.7)		
Lecithin		0.5 Gm.
Tween 80		3 Gm.
Medium I	*q.s. ad.*	100 ml.
Medium VI (pH 6.7)		
Tween 20[e]		3 Gm.
Medium I	*q.s. ad.*	100 ml.
Medium VII (pH 6.7)		
Lecithin		0.5 Gm.
Tween 20		3 Gm.
Medium I	*q.s. ad.*	100 ml.
Medium VIII (pH 7.1)		
Fluid thioglycollate medium, dehydrated[g]		2.93 Gm.
Purified water	*q.s. ad.*	100 ml.
Medium IX (pH 7.0)		
Tween 80		3 Gm.
Medium VIII	*q.s. ad.*	100 ml.
Medium X (pH 7.0)		
Lecithin		0.5 Gm.
Tween 80		3 Gm.
Medium VIII	*q.s. ad.*	100 ml.
Medium XI (pH 7.0)		
Tween 20		3 Gm.
Medium VIII	*q.s. ad.*	100 ml.
Medium XII (pH 7.0)		
Lecithin		0.5 Gm.
Tween 20		3 Gm.
Medium VIII	*q.s. ad.*	100 ml.
Medium XIII (pH 7.1)		
Glycerin		4 Gm.
Medium VIII	*q.s. ad.*	100 ml.
Medium XIV (pH 7.1)		
Lecithin		0.5 Gm.
Glycerin		4 Gm.
Medium VIII	*q.s. ad.*	100 ml.
Medium XV (pH 6.7)		
Tween 80		5 Gm.
Medium I	*q.s. ad.*	100 ml.
Medium XVI (pH 6.6)		
Tween 80		10 Gm.
Medium I	*q.s. ad.*	100 ml.
Medium XVII (pH 6.7)		
Tween 20		5 Gm.
Medium I	*q.s. ad.*	100 ml.
Medium XVIII (pH 6.6)		
Tween 20		10 Gm.
Medium I	*q.s. ad.*	100 ml.

[a] Difco Laboratories, Inc., Detroit, Mich. [b] Colgate Palmolive Co., New York, N. Y. [c] Lecithin, (Ex Ovo Soluble) Pfansteihl Laboratories, Inc., Waukegan, Ill. [d] Polyoxyethylene (20) sorbitan mono-oleate, Atlas Powder Co., Wilmington, Del. [e] Polyoxyethylene (20) sorbitan monolaurate, Atlas Powder Co., Wilmington, Del. [f] All media containing nutrient broth, dehydrated, were sterilized by autoclaving at 121° for 15 minutes. [g] All media containing fluid thioglycollate medium dehydrated were autoclaved at 121° for 20 minutes.

test, the cultures were shaken by hand approximately 1 minute to break up clumps and allowed to stand for at least 15 minutes. Dilutions (1:10 and 1:1000) of the 24-hour cultures of the two strains were made using nutrient broth, and the approximate number of viable bacteria were determined by the pour-plate technique. The 1:10 and 1:1000 dilutions of the 24-hour cultures contained approximately 10^8 and 10^6 bacteria per ml., respectively, as noted after incubating the plates at 37° for 48 hours.

Preparation of the Antibacterial Agent–Medium Mixtures.—Four milliliters of each of the various test media was placed in culture tubes (approximately 15 cm. in length and 13 mm. inside diameter) and sterilized. Stock solutions of several concentrations of the antibacterial agents were prepared as aqueous solutions. One milliliter of a sterile stock solution of the antibacterial agent was added to a tube containing 4 ml. of the sterile medium. This was done for each concentration of the antibacterial agent tested and also for each medium being used. The tubes were then shaken by hand to insure a uniform mixture.

Preparation of the Controls.—Controls were employed to determine bacterial contamination (negative controls) and to determine whether the media were capable of supporting the growth of *Ps. aeruginosa* (positive controls). Media prepared with the antibacterial agent but without the presence of *Ps. aeruginosa* were employed as negative controls. Media prepared without the antibacterial agents but with *Ps. aeruginosa* were employed as positive controls.

Procedure.—One-tenth milliliter of the 1:10 dilution of each strain of *Ps. aeruginosa* was added separately to a tube containing a single concentration of the sterile antibacterial agent in the individual medium being tested. This was repeated for each concentration of the antibacterial agent in each medium being tested. Each tube was shaken by hand to insure an even distribution of the bacteria. After 1 hour of contact at 24°, 0.5 ml. was transferred into tubes containing 4.5 ml. of the subculture medium, and the mixture was shaken by hand. After 24 hours of contact at 24°, another transfer of 0.5 ml. was made into tubes containing 4.5 ml. of the subculture medium. The same procedure was repeated with the 1:1000 dilution of the strain used and with the two dilutions of the other strain. All tubes were incubated at 37° for a period of 7 days and observed for the presence or absence of growth. The procedure was repeated to verify the findings.

Experiment II

Purpose.—As previously indicated in the evaluation of the antibacterial activity of chemical agents intended for use as preservatives in ophthalmic solutions, an important consideration is the time required for such agents to kill the bacteria after contact (sterilizing time). *Experiment II* was therefore devised to determine whether the antibacterial agent is fast-acting or slow-acting. This experiment was also designed to show the necessity of developing subculture media containing suitable inactivating agents.

Preparation of the Inoculum.—A 1:10 dilution of each of the 24-hour-old cultures of the 13 strains of *Ps. aeruginosa* was used in this study. Each was prepared as described previously.

Preparation of the Media.—Tubes containing 4.5 ml. of each of the various test media described previously were prepared and sterilized.

Preparation of the Antibacterial Solutions.—Tubes containing 5 ml. of the sterile aqueous antibacterial solutions to be tested were prepared.

Preparation of Controls.—Controls were employed to determine bacterial contamination (negative controls) and to determine whether the media were capable of supporting the growth of *Ps. aeruginosa* (positive controls). Tubes containing 5 ml. of the antibacterial solutions, without the presence of *Ps. aeruginosa*, were employed as negative controls. Solutions prepared without the antibacterial agent but containing *Ps. aeruginosa* were employed as positive controls.

Procedures.—One-tenth milliliter of a 1:10 dilution of each strain of *Ps. aeruginosa* was separately added to a tube containing 5 ml. of the sterile antibacterial solution. This was repeated for all 13 strains. Each tube of the antibacterial agent-bacteria mixture was then shaken by hand. After 1-hour of contact at 24°, 0.5 ml. was transferred into the tube containing 4.5 ml. of the subculture medium. This was then shaken by hand. After 24 hours of contact at 24°, another transfer of 0.5 ml. was made into 4.5 ml. of the subculture medium. All subculture tubes were incubated at 37° for a period of 7 days and observed for the presence or absence of growth. The procedure was repeated to verify the findings.

Experiment III

Purpose.—The purpose of this experiment was similar to that of *Experiment II*, except that the exact sterilizing time (as noted under *Procedure*) was determined.

Preparation of the Inoculum.—Two dilutions (1:10 and 1:1000) of the 24-hour-old cultures of 13 different strains of *Ps. aeruginosa* were used in this experiment. They were prepared as previously described.

Preparation of the Medium.—Four and one-half milliliters of the medium, which had shown the maximum inactivation of the antibacterial agent being tested, was employed.

Preparation of the Antibacterial Solutions.—Tubes containing 5 ml. of the sterile antibacterial solution to be tested were prepared.

Preparation of Controls.—Negative and positive controls were prepared.

Procedure.—The procedure was similar to that employed in *Experiment II*, except for changes in the contact times. If the results of *Experiment II* showed the antibacterial agent to be effective against *Ps. aeruginosa* within 1 hour, *i.e.*, no growth in the subculture tubes containing the 1-hour transfer, then the agent was tested at contact times of 15, 30, 45, and 60 minutes. If the antibacterial agent was shown to be effective between 1 and 24 hours, *i.e.*, no growth in the subculture tubes containing the 24-hour transfers, tests were carried out at 3, 6, 9, and 12 hours of contact. If necessary 15, 18, and 21 hours of contact also were employed. If the antibacterial agent was shown to be ineffective after 24 hours of contact, further studies were not considered warranted.

Experiment IV

Purpose.—In *Experiment III*, the sterilizing time of each chemical agent employed as a preservative in ophthalmic solutions was determined against *Ps. aeruginosa*. Obviously, the sterilizing time must be based on the total destruction of all bacteria. In *Experiment III*, only 0.5 ml. of the antibacterial agent–bacteria mixtures was transferred to the subculture medium. The purpose of this experiment was to determine whether this volume of inoculum was as effective as would be transplants of larger volumes.

Preparation of the Inoculum.—The most resistant strains of *Ps. aeruginosa*, as noted in *Experiment III*, were selected and used in this experiment. A 1:10 and a 1:1000 dilution of the 24-hour-old cultures of the selected strains were used. They were prepared as described previously.

Preparation of the Medium.—The medium used in *Experiment III* was employed here. Four and one-half milliliters, 9 ml., 18 ml., and 45 ml. of the medium, respectively, were placed separately in appropriate containers and sterilized.

Preparation of the Antibacterial Solutions.—The concentration of the antibacterial solution used in this study depended upon the strains of *Ps. aeruginosa* which were selected. A series of four tubes containing 5 ml. of the sterile antibacterial solution was prepared for each dilution of each strain of *Ps. aeruginosa* employed in this experiment.

Preparation of the Controls.—Controls were prepared as described in *Experiment II*.

Procedure.—The contact time used in this experiment was the same as that which revealed no growth immediately following a contact time period which had shown growth in the subculture medium for the strains selected. On the day of the experiment, 0.1 ml. of each of the diluted strains was added separately to each tube in the series containing the antibacterial solution. This was repeated for each strain used in this experiment. Transfers were made from the series of tubes at the selected contact time, using a 0.5 ml., 1 ml., 2 ml., and 5 ml. inoculum into 4.5 ml., 9 ml., 18 ml., and 45 ml. of subculture medium, respectively. The containers of subculture medium–antibacterial agent–bacteria mixtures were shaken by hand. All containers of this subculture medium of the antibacterial agent–bacteria mixture were incubated at 37° for a period of 7 days and observed for the presence or absence of growth. The entire procedure was repeated to verify the findings.

Antibacterial Agents Studied

The antibacterial agents studied for their effectiveness as preservatives for ophthalmic solutions against *Ps. aeruginosa* were as follows: chlorobutanol,[1] benzalkonium chloride,[2] thimerosol,[3] combinations of methylparaben and propylparaben,[4] phenylmercuric nitrate,[5] phenylethyl alcohol,[6] and polymyxin B sulfate.[7] Aqueous solutions of these agents were prepared, sterilized by appropriate procedures, and assayed for content of active ingredient (22).

Fig. 1.—Appearance of the rabbit eye infected with *Ps. aeruginosa*.

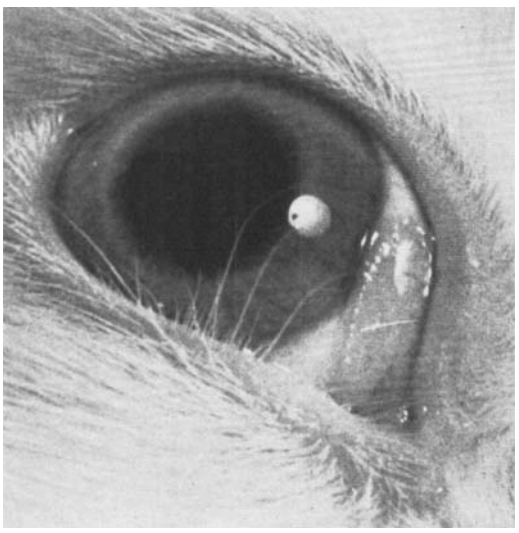

Fig. 2.—Appearance of the normal rabbit eye.

In Vivo Method

Purpose.—The purpose of the *in vivo* study is to prove that (*a*) the results obtained by employing *Experiment III* (*in vitro* method) for noting the efficiency of the antibacterial agents are valid, *i.e.*, the bacteria are incapable of growing in the subculture medium and accordingly are also incapable of producing an ocular infection *in vivo;* and (*b*) the inactivating media utilized in this study are adequate.

Preparation of the Inoculum.—The inoculum of *Ps. aeruginosa* was prepared as previously described. For the most part, the strains of *Ps. aeruginosa* which proved to be the most resistant to the antibacterial agents in the *in vitro* experiments were employed in this study. Occasionally an intermediate strain, with regard to resistance toward the antibacterial agent, and also a mixture of resistant strains were employed. The mixture of resistant strains was prepared on the day of the test by mixing 1 ml. of each of the respective 24-hour-old nutrient broth cultures in a sterile tube and

[1] Chlorobutanol, Hydrous, Merck and Co., Inc., Rahway, N. J.
[2] Zephiran Chloride, Winthrop Laboratories, Inc., New York, N. Y.
[3] Mann Fine Chemicals, Inc., New York, N. Y.
[4] Heyden Chemical Corp., New York, N. Y.
[5] Metalsalts Corp., Hawthorne, N. J.
[6] S. B. Penick and Co., Inc., New York, N. Y.
[7] Chas. Pfizer and Co., New York, N. Y.

TABLE II.—HIGHEST CONCENTRATION OF PRESERVATIVES INACTIVATED BY VARIOUS MEDIA

Medium	Preservative: Chlorobutanol, %	Benzalkonium Chloride	Thimerosol	Methylparaben, % / Propylparaben, %		Phenylmercuric Nitrate	Phenylethyl Alcohol, %	Polymyxin B Sulfate, Units
I	0.4	1:30,000	[b]	0.14	0.02	[b]	0.2	31.25
II	...	1:30,000	...	...	...	[b]	...	15.6
III	...	1:2500	...	...	...	...	...	2000[a]
IV	...	1:1000[a]	[b]	...	...	...	...	31.25
V	...	1:1000[a]	[b]	...	...	1:50,000	...	31.25
VI	...	1:1000[a]	...	...	...	...	...	31.25
VII	...	1:1000[a]	...	...	...	...	...	31.25
VIII	...	...	1:20,000	...	...	1:6250	...	...
IX	...	...	1:20,000	...	...	1:6250	...	...
X	...	...	1:2500[a]	...	...	1:3125[a]	...	...
XI	...	...	1:20,000	...	...	1:6250	...	...
XII	...	...	1:2500[a]	...	...	1:3125[a]	...	...
XIII	...	...	1:20,000	...	...	1:6250	...	...
XIV	...	...	1:2500[a]	...	...	1:3125[a]	...	...
XV	0.5	...	...	0.2[a]	0.04[a]	...	0.5	...
XVI	0.6[a]	...	...	0.2[a]	0.04[a]	...	1.0[a]	...
XVII	0.6[a]	...	...	0.2[a]	0.04[a]	...	0.5	...
XVIII	0.6[a]	...	...	0.2[a]	0.04[a]	...	1.0[a]	...

[a] Highest concentration tested. [b] Growth was not evident in this medium when the preservative was in a concentration of 1:50,000 (the smallest concentration studied).

proceeding as when a single strain of *Ps. aeruginosa* was used.

Preparation of the Antibacterial Agent-Bacteria Mixtures.—One-tenth milliliter of the dilution of the strain or mixture of strains was added to 5 ml. of the sterile preservative solution being investigated. The tube was shaken by hand for approximately 15 seconds and allowed to remain at 24° for a specific contact time, each time period depending on the results obtained in the *in vitro* experiments for the antibacterial agent being studied.

Preparation of the Controls.—A sterile solution of the preservative was employed as a positive control, not only for accidental contamination, but also to detect any action and untoward reaction the preservative might have on the rabbit's eye. A dilution of *Ps. aeruginosa*, containing approximately 10,000 bacteria per ml., was employed as a negative control to determine pathogenicity of *Ps. aeruginosa* in the rabbit's eye.

Procedure.—Two contact time periods were employed for each antibacterial agent-bacteria mixture studied. The contact time periods were selected, as mentioned previously, from the findings of the *in vitro* experiments for the various antibacterial agents tested. The first contact time period selected was the longest period of time which still showed growth of the bacteria in the subculture medium; also the contact time period immediately following the first contact time period was chosen. Obviously, the latter contact time period would have shown no growth of the bacteria in the subculture medium. The first exposure period was employed to prove that when growth of *Ps. aeruginosa* occurs in the subculture medium, an infection could also be produced in the rabbit's eye. The second contact time period was utilized to fulfill the purpose of the *in vivo* experiment.

Usually six rabbits[8] were employed for each antibacterial agent studied. The rabbits were placed by hand in individual cages which were thoroughly cleaned with a disinfectant and live steam after each experiment. The antibacterial agent–bacteria mixture was injected intracorneally into the rabbit's eye. Approximately 0.01 ml. was injected from a one-quarter-ml. tuberculin syringe[9] graduated in 0.01 ml. One-quarter-inch 27-gauge needles[9] were employed. Approximately 5 minutes before each injection, two drops of a 2% butacaine sulfate[10] solution were placed in the conjunctival sacs of each rabbit.

At the first contact time period selected an intracorneal injection of the antibacterial agent–bacteria mixture was given into one eye of each of two rabbits. At the next contact time, another intracorneal injection was given into the other eye of each of the same two rabbits. A third rabbit received an injection of the sterile antibacterial solution (positive control) in one eye and an injection of the diluted bacteria (negative control) in the other eye. The rabbits were observed for a period of 7 days for evidence of ocular infections (see Fig. 1). Figure 1 shows the appearance of an eye infected with *Ps. aeruginosa* 5 days after the injection. Figure 2 shows the appearance of a normal eye. Most eyes infected with *Ps. aeruginosa* were completely closed within 7 days after the injection.

As a further control, swabs were periodically taken of any infected eyes to recover and identify the presence of *Ps. aeruginosa*.

The above procedure was repeated, employing another strain or a mixture of strains of *Ps. aeruginosa*.

RESULTS

From the data obtained from *Experiments I* and *II* employing the different antibacterial agents as preservatives (Tables II and III), the following media were selected to be employed as the inactivating media in *Experiments III* and *IV:* Medium XVIII for chlorobutanol, Medium V for benzalkonium chloride, Medium X for thimerosol, Medium XVIII for combinations of methylparaben and propylparaben, Medium X for phenylmercuric

[8] Male white New Zealand rabbits, Huntingdon Farms, Philadelphia, Pa.

[9] Becton, Dickinson and Co., Rutherford, N. J.

[10] Butyn Sulfate, Abbott Laboratories, Chicago, Ill.

nitrate, and Medium III for polymyxin B sulfate.

Media XVI and XVIII were found to be the best neutralizing media for phenylethyl alcohol in *Experiment I*. Since the sterilizing time for phenylethyl alcohol was found to be greater than 24 hours, further experiments were not carried out on this chemical in *Experiment II*.

In Table IV a summary of the results obtained in *Experiment III* is presented. The sterilizing times which are listed against *Ps. aeruginosa* for each of the chemical agents are those which have been obtained under severe *in vitro* test conditions (dilution of bacterial inoculum was 1:10). They indicate the time required to kill all of the strains of *Ps. aeruginosa* employed in this study.

The results of *Experiment IV* are not recorded here, but in all cases they indicate that 0.5 ml. was adequate as the volume of antibacterial agent–bacteria mixture used as the transfer in *Experiments II* and *III*.

The results of the *in vivo* experiments indicate that they are in agreement in all instances with the *in vitro* findings of *Experiment III, i.e.*, those solutions which produced growth following transfer into subculture media produced ocular infections; those which did not produce growth did not produce ocular infections.

DISCUSSION

In the evaluation of the effectiveness of antibacterial agents used as preservatives in ophthalmic solutions against *Ps. aeruginosa*, the time required for such an agent to kill the bacteria must be known. The experiments employed in these studies determine the sterilizing time for each antibacterial agent against *Ps. aeruginosa*. In addition, other important factors are evident in the experiments previously described which are not contained in those procedures in current use or those employed in the past.

One advantage is that the procedures do not rely upon a dilution technique to differentiate between bacteriostatic and bactericidal effects of the antibacterial agents being tested. Inactivating agents were employed for this purpose. The latter, incorporated in the subculture media, function by inactivating or neutralizing the antibacterial properties of the chemical agents employed for their preservative activity.

Another advantage of the test procedures employed in this study is that they establish the inactivating or neutralizing ability of the subculture media toward the antibacterial activity of the chemical agents studied. Such data have not been reported previously by the various workers in the field.

In the evaluation of the effectiveness of antibacterial preservatives, it is not only important to determine the time required for sterilization, but it is also necessary to determine whether the bactericidal effect was complete. A method which evaluates the destruction of bacteria but neglects to determine whether a total kill has occurred may

TABLE III.—NUMBER OF STRAINS OF *Ps. aeruginosa* WHICH DISPLAYED GROWTH IN BEST TEST MEDIA AFTER CONTACT WITH PRESERVATIVES[a]

Preservative	Contact Time, hr.	Medium I	XV	XVI	XVII	XVIII		
Chlorobutanol, 0.5%	1	13	13	13	13	13		
	24	0	0	0	0	0		
		Medium I	II	III	IV	V	VI	VII
Benzalkonium chloride, 1:5000	0.5	2	1	1	3	4	3	4
	24	0	0	0	0	0	0	0
		Medium I	IV	V	VIII	IX		
Thimerosol, 1:5000	1	0	0	0	3	3		
	24	0	0	0	0	0		
		Medium X	XI	XII	XIII	XIV		
Thimerosol, 1:5000	1	13	3	13	3	3		
	24	0	0	0	0	0		
		Medium I	XV	XVI	XVII	XVIII		
Methylparaben, 0.2%	1	2	3	6	3	6		
Propylparaben, 0.04%	24	0	0	0	0	0		
		Medium I	IV	V	VIII	IX		
Phenylmercuric nitrate,	1	0	0	0	0	3		
1:10,000	24	0	0	0	0	0		
		Medium X	XI	XII	XIII	XIV		
Phenylmercuric nitrate,	1	7	2	6	0	2		
1:10,000	24	0	0	0	0	0		
		Medium I	XV	XVI	XVII	XVIII		
Phenylethyl alcohol, 0.5%	1	13	13	13	13	13		
	24	13	13	13	13	13		
		Medium I	II	III	IV	V	VI	VII
Polymyxin B sulfate, 1000	1	0	0	13	0	0	0	0
units/ml.	24	0	0	0	0	0	0	0

[a] Thirteen strains tested.

prove to be in error. In the experiments employed in this investigation, the adequacy of the volume of the antibacterial agent–bacteria mixture transferred to the subculture media has been evaluated. This has not usually been taken into consideration.

It was noted that the results of the *in vivo* test agreed at all times with the results of the *in vitro* procedures. This was additional evidence of the effectiveness of the inactivating media employed for the various antibacterial chemical agents under study.

An antibacterial agent intended for use as a preservative in ophthalmic solutions should have quick-acting bactericidal activity. There is no exact definition for the term *quick-acting*. In this discussion, an antibacterial agent will arbitrarily be considered to possess *quick-acting* bactericidal activity against *Ps. aeruginosa* if the sterilizing time of that agent was 1 hour or less under the extreme test conditions employed in this study.

Chlorobutanol is not a quick-acting antibacterial preservative aginst *Ps. aeruginosa*. A 12-hour period of contact was required for a 0.5% solution of chlorobutanol, which is the concentration most commonly employed to produce a bactericidal effect in marketed multiple-dose ophthalmic solutions. Lawrence (16) and Riegelman, *et al.* (17), also reported chlorobutanol to be slowly bactericidal against *Ps. aeruginosa*. A 10% Tween 20 nutrient broth medium was found the most effective inhibiting medium for chlorobutanol. The probable mechanism of action whereby Tween 20 acts is probably a binding or association reaction between the chlorobutanol and the Tween.

Of all the antibacterial agents investigated in this study, benzalkonium chloride, in a concentration of 1:5000, revealed the quickest-acting bactericidal activity against *Ps. aeruginosa*. The results obtained for benzalkonium chloride are in agreement with those of Lawrence (16), but they disagree with those of Riegelman, *et al.* (17), who reported a longer sterilizing time against *Ps. aeruginosa*. This may be due to the strains of *Ps. aeruginosa* employed. The subculture medium found to have the greatest neutralizing activity against benzalkonium chloride contained polysorbate 80 (Tween 80) and lecithin as the inactivating agents. Both of these compounds inactivate the antibacterial activity of benzalkonium chloride; however, the mechanisms of inactivation are different. The possible mechanism of action of the polysorbate 80 is a binding or association reaction with the benzalkonium chloride, whereas the negative charge of lecithin is probably responsible for the inactivating role it plays.

TABLE IV.—STERILIZING TIMES AGAINST *Ps. aeruginosa* (10^8) FOR CHEMICAL AGENTS COMMONLY EMPLOYED AS PRESERVATIVES IN OPHTHALMIC SOLUTIONS

Chemical Agents	Concn.	Sterilizing Time
Chlorobutanol	0.7%	9 hr.
Chlorobutanol	0.5%	12 hr.
Benzalkonium chloride	1:5000	45 min.
Benzalkonium chloride	1:10,000	9 hr.
Thimerosol	1:5000	6 hr.
Thimerosol	1:10,000	9 hr.
Methylparaben and propylparaben	0.2% 0.04%	3 hr.
Methylparaben and propylparaben	0.18% 0.02%	6 hr.
Phenylmercuric nitrate	1:10,000	6 hr.
Phenylmercuric nitrate	1:20,000	6 hr.
Phenylethyl alcohol	0.5%	[a]
Polymyxin B sulfate	2000 units/ml.	12 hr.
Polymyxin B sulfate	1000 units/ml.	18 hr.

[a] Sterilization not effected in 24 hours.

The results indicate that thimerosol, in the highest concentration (1:5000) usually employed as an antibacterial preservative in marketed ophthalmic solutions, is too slow-acting in its bactericidal effect (6 hours) against *Ps. aeruginosa*. The inactivating medium employed to neutralize the antibacterial action of thimerosol was fluid thioglycollate medium containing polysorbate 80 and lecithin. The former, by itself, has had general acceptance as the inactivating medium for the antibacterial organic mercurials, there being an interaction between the mercurial ion and the —SH groups of the thioglycollic acid. However, it was found to be less effective than the combination of substances employed in our inactivating medium.

A combination of 0.2% of methylparaben and 0.04% propylparaben possessed a sterilizing time of 3 hours against *Ps. aeruginosa*. However, this concentration is not usually employed in ophthalmic solutions. In a concentration more commonly employed, 0.18% methylparaben and 0.02% propylparaben, the sterilization time required was 6 hours. A Tween 20–nutrient broth medium was employed as the inactivating medium for the antibacterial activity of the parabens. Tween 20 as the major inactivator probably functions by the same mechanism of action as that discussed under chlorobutanol.

The results indicate that phenylmercuric nitrate acts too slowly as a bactericide against *Ps. aeruginosa* for use as an antibacterial preservative in ophthalmic solutions, the sterilizing time being 6 hours. The findings in *Experiments I* and *II* indicated that polysorbate 80 and lecithin in fluid thioglycollate medium was most effective in neutralizing the antibacterial activity of phenylmercuric nitrate and even better than fluid thioglycollate medium by itself. This observation was also made by Riegelman, *et al.* (17). The increase in the inactivating ability of fluid thioglycollate medium when it contains lecithin solubilized by polysorbate 80 is probably because of the interaction of the oppositely charged molecules of lecithin and phenylmercuric nitrate.

A 0.5% solution of phenylethyl alcohol did not have sterilizing power when in contact with *Ps. aeruginosa* for a period of 24 hours and must therefore be considered to be a very inferior antibacterial preservative against this organism.

Polymyxin B sulfate was found to be only slowly bactericidal against *Ps. aeruginosa* and is therefore unsuitable as an antibacterial preservative in multidose ophthalmic solutions. This conclusion does not agree with that of Riegelman, *et al.* (17). However, Riegelman's results could not be duplicated when employing his technique, the inactivating medium used by him and his workers, and utilizing the strains of *Ps. aeruginosa* employed in this investigation. A possible explanation for this discrepancy may be the resistance of the strains of *Ps. aeruginosa* employed toward polymyxin B sulfate. Attention is directed to the fact that Riegelman, *et al.* (17),

did not employ many strains of *Ps. aeruginosa* in their experiments. It was observed that lecithin, suspended with the aid of glycerin in nutrient broth, was most effective in inactivating polymyxin B sulfate. The possible mechanism of action is similar to that given under benzalkonium chloride. It was also noted that nutrient broth containing lecithin solubilized by either Tween 20 or Tween 80 was not as effective as the above-mentioned inactivating medium. This observation was also noted by Riegelman, *et al.* (17), and Bliss and Worth (23). A possible explanation may be that the effective charge of the lecithin molecule is reduced when it is solubilized by the Tweens. This reduction in the inhibiting action of lecithin by the Tweens was not observed with benzalkonium chloride. The Tweens, by themselves, were capable of reducing the antibacterial action of benzalkonium chloride, while they had no such effect on polymyxin B sulfate.

SUMMARY

1. Various *in vitro* studies were carried out to determine the antibacterial effectiveness of various chemical agents to be used as preservatives in ophthalmic solutions against 13 strains of *Ps. aeruginosa*. Methods were devised to (*a*) differentiate between the bacteriostatic and bactericidal properties of the chemical preservatives by formulating suitable inactivating media, (*b*) determine the sterilizing time for each chemical preservative, and (*c*) determine whether the volume of inoculum taken for sampling in the experiment was adequate.

2. An *in vivo* procedure was employed in evaluating the chemical agents to note whether findings would be in agreement with the final results obtained in the *in vitro* studies.

3. The following antibacterial agents used or recommended for use in ophthalmic solutions were thoroughly investigated: chlorobutanol, benzalkonium chloride, thimerosol, combinations of methylparaben and propylparaben, phenylmercuric nitrate, phenylethyl alcohol, and polymyxin B sulfate. Of these, only benzalkonium chloride possessed a sterilizing time against *Ps. aeruginosa* of less than 1 hour.

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Effect of Certain Drugs on Perfused Human Placentas II

Vasodilators

By HENRY P. CIUCHTA and RONALD F. GAUTIERI

Perfusion studies conducted on full term human placentas produced the following results: marked vasodilation with isosorbide dinitrate, isoproterenol hydrochloride, nitroglycerin and papaverine hydrochloride, slight vasodilation, and vasoconstriction with cyclandelate and isoxsuprine hydrochloride. Except for alcohol, which produced a slight decrease in the volume flow rate through the placenta, the solvents employed with these drugs had no effect on placental vasculature.

During the past 40 years the action of various agents on placental vasculature has been studied by numerous investigators, including Kosakae (1), Ueda (2), von Euler (3), Schmitt (4), Eliasson and Astrom (5), and Astrom and Samelius (6). The results of these experiments are rather inconsistent. This undoubtedly is partly due to the employment of different experimental procedures and the inability at times to obtain placentas within a short time after delivery. More than likely, the

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did not employ many strains of *Ps. aeruginosa* in their experiments. It was observed that lecithin, suspended with the aid of glycerin in nutrient broth, was most effective in inactivating polymyxin B sulfate. The possible mechanism of action is similar to that given under benzalkonium chloride. It was also noted that nutrient broth containing lecithin solubilized by either Tween 20 or Tween 80 was not as effective as the above-mentioned inactivating medium. This observation was also noted by Riegelman, *et al.* (17), and Bliss and Worth (23). A possible explanation may be that the effective charge of the lecithin molecule is reduced when it is solubilized by the Tweens. This reduction in the inhibiting action of lecithin by the Tweens was not observed with benzalkonium chloride. The Tweens, by themselves, were capable of reducing the antibacterial action of benzalkonium chloride, while they had no such effect on polymyxin B sulfate.

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During the past 40 years the action of various agents on placental vasculature has been studied by numerous investigators, including Kosakae (1), Ueda (2), von Euler (3), Schmitt (4), Eliasson and Astrom (5), and Astrom and Samelius (6). The results of these experiments are rather inconsistent. This undoubtedly is partly due to the employment of different experimental procedures and the inability at times to obtain placentas within a short time after delivery. More than likely, the

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major reason for varying results is the sensitivity of the placenta itself.

Schmitt (4), besides demonstrating that the placenta is devoid of innervation, showed that histamine, posterior pituitary extract, and barium chloride constricted placental vasculature, while amyl nitrite caused a dilatation and adrenaline produced no effect. von Euler (3) conducted extensive studies on "nerve free" vessels of the placenta and found only a constrictor action with epinephrine, acetylcholine, histamine, pitressin, and barium chloride. He postulated that these drugs may act on some intermediate mechanism of the contractile element.

In an earlier work (7) von Euler observed that acetylcholine constricted pulmonary vessels in the rabbit. From these investigations (3, 7) he proposed that a correlation may exist between the responses of placental and pulmonary vessels, since arteries of both organs carry venous blood and are structurally adapted to withstand moderate pressures. Smith and Coxe (8) submitted supporting evidence in observing that pulmonary vessels in cat, dog, swine, and human lungs were constricted with epinephrine, acetylcholine, and histamine.

Experiments conducted by Dornhorst and Young (9) on placental vessels of guinea pig *in situ* indicated that adrenaline and noradrenaline caused a constriction of these vessels by a direct action, thus impairing placental circulation.

Eliasson and Astrom (5) observed vasoconstriction in placental vessels with epinephrine, norepinephrine, dihydroergotamine, acetylcholine, and histamine. Astrom and Samelius (6), in addition to testing the effects of lysergic acid, chlorpromazine, tryptamine, yohimbine, mescaline, phentolamine, phenbenzamine, reserpine, ganglionic blocking agents, and heparin, established the potent vasoconstrictor activity of serotonin on placental vasculature.

Previously in this laboratory (10) it was demonstrated that the vasculature of the placenta undergoes various degrees of vasoconstriction in the presence of narcotic analgesics, serotonin, and relaxin.

Since the actions of many vasoconstrictors on placental vessels have been intensively investigated, a study of the effects of vasodilators on this preparation is indicated. Proof exists that morphine (10) and histamine (5), which usually elicit vasodilation in man, cause vasoconstriction in placental vessels. Therefore, there is a need to determine whether certain vasodilators may also follow a similar pattern of producing an opposite effect on placental vasculature. The placenta provides a valid technique for ascertaining the action of musculotropic drugs because it is devoid of nervous tissue.

Therefore, it was the purpose of this investigation to observe the effects of clinically proved vasodilators alone on placental vasculature, and in certain cases, in the presence of drug induced spasm.

MATERIALS AND METHODS

Full term human placentas were obtained from the hospital 5–15 minutes after normal delivery. Each was transported to the laboratory in a container of 38° preheated Tyrode's solution.

The preparation was flushed free of blood by injecting 10% sodium citrate solution into one of the arteries. Cannulas were inserted into the vein and one artery of the umbilical cord 5–10 cm. from the stalk of the placenta and the remaining umbilical artery was ligated. The whole preparation was then placed in a perfusion chamber containing aerated Tyrode's solution which had a pH range of 7.2 to 7.4 and was maintained at a constant 37° by a Bronwill thermoregulator. The arterial cannula was connected with rubber tubing to a hydrostatic reservoir which was adjusted to yield pressures ranging between 40–60 mm. Hg. The reservoir contained Tyrode's solution which was modified by the addition of 0.525% polyvinylpyrrolidone (Plasdone C). The venous outflow, ranging between 10–60 ml./minute, was measured directly by means of a graduated cylinder and not reperfused.

Two placentas were discarded because the outflow per minute at the onset of the experiment was far below average; it was considered that the volume changes would not be of significant value.

TABLE I.—THE EFFECTS OF DRUGS ON VOLUME FLOW IN PERFUSED HUMAN PLACENTAS

	Composite Results					
Drug	No. of Prepns.	Dose	Vol. Flow (Range), % Increase	Decrease	Av. %	S. D.
Cyclandelate	4	1–4 mg.	11–19	...	15.5	2.6
Cyclandelate	11	1–4 mg.	...	5–15	10.2	3.5
Isoxsuprine	10	1–10 mg.	5–19	...	9.4	4.0
Isoxsuprine	5	1–10 mg.	...	5–11	7.0	2.3
Isosorbide	5	1–2 mg.	25–116	...	49.6	37.4
Isoproterenol	11	2.0 mg.	5–84	...	41.7	28.6
Nitroglycerin (spirit)	5	0.2 ml.	18–159	...	73.4	67.0
Nitroglycerin (aqueous)	6	1–4 mg.	5–36	...	20.3	11.6
Papaverine	9	4–5 mg.	5–65	...	30.4	15.0
Serotonin	9	50–100 mcg.	...	7–81	28.8	24.6

In 85 successful experiments the average duration of each preparation was between 2 and 3 hours.

The following drugs were injected into the rubber tubing adjacent to the arterial cannula in a volume of distilled water not exceeding 1 ml.: cyclandelate,[1] isoxsuprine hydrochloride,[2] isosorbide dinitrate,[1] isoproterenol hydrochloride,[3] nitroglycerin (alcoholic and aqueous vehicles), papaverine hydrochloride, and 5-hydroxytryptamine creatinine sulfate (serotonin). In addition, alcohol and propylene glycol were injected to observe if these vehicles elicited action of their own.

RESULTS

Cyclandelate.—Cyclandelate in doses of 1–4 mg. (four preparations) produced a slight vasodilation of placental vessels. The increase in volume outflow ranged from 11–19% (Table I). However, in 11 preparations there was a decrease in outflow ranging from 5–15% (Table I). After cyclandelate administration, three preparations yielded a change in volume outflow below 5% and were not counted.

The administration of serotonin after pretreatment of a preparation with cyclandelate elicited a typical vasoconstrictor effect. Isoproterenol injection caused an increase in volume outflow after a volume decrease due to cyclandelate.

Isoxsuprine.—The administration of isoxsuprine hydrochloride (10 cases) in doses of 1–10 mg. produced an increase in volume outflow of 5–19% (Table I). In five cases there was a 5–11% decrease in the outflow rate (Table I). A change in the outflow rate below 5% was recorded in 10 preparations.

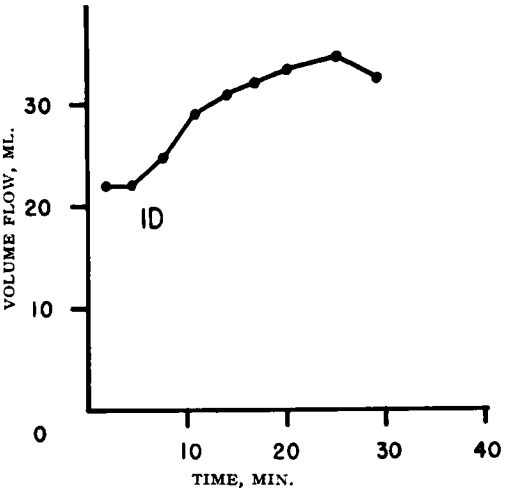

Fig.1—Effect of isosorbide dinitrate (1 mg.) on volume flow rate in vessels of the perfused human placenta.

After a decrease or increase in outflow rate due to isoxsuprine, the administration of serotonin in certain cases brought about vasoconstriction, while subsequent injections of papaverine increased the volume outflow.

Isosorbide.—In a total of five experiments isosorbide dinitrate in doses of 1–2 mg. produced an increase in volume outflow (Fig. 1). The range of outflow was increased 25–116% (Table I). Only one preparation yielded a change in outflow rate below 5%.

The subsequent administration of nitroglycerin caused an even greater increase in the volume outflow in certain preparations.

Isoproterenol.—The administration of 2 mg. of isoproterenol hydrochloride (11 preparations) brought about a powerful vasodilation (Fig. 2) which was of a long duration and not increased by the subsequent injection of cyclandelate in certain cases. The outflow rate increased from 5–84% (Table I). Results obtained from three preparations were under 5%.

Nitroglycerin.—In five cases dramatic increases in volume outflow (Fig. 3) were obtained with the administration of 0.2 ml. of nitroglycerin spirit (92% alcohol as the vehicle). The per cent increase in volume outflow ranged from 18–159 (Table I).

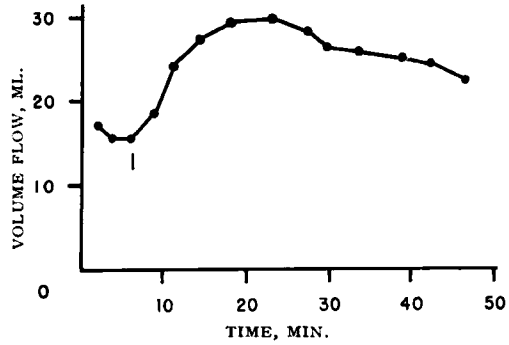

Fig. 2—Effect of isoproterenol (2 mg.) on volume flow rate in vessels of the perfused human placenta.

The administration of 1–4 mg. of nitroglycerin (aqueous vehicle) produced an increase in volume outflow in six cases, having a range of 5–36% (Table I). All preparations yielded increases of 5% or more.

The administration of serotonin in certain preparations following alcoholic nitroglycerin brought about a slight and transient vasoconstriction. Treatment of a preparation with isosorbide after aqueous nitroglycerin caused a significant increase (32%) in volume outflow.

Papaverine.—In nine preparations doses of 4–5 mg. of papaverine hydrochloride produced an increase in volume outflow ranging from 5–65% (Table I). The change in outflow rate of two preparations was under 5%.

The administration of barium chloride (Fig. 4) and serotonin (Fig. 5) following papaverine injection elicited vasoconstriction.

Serotonin.—The administration of serotonin in 50–100-mcg. doses in nine cases evoked a marked decrease in volume outflow ranging from 7–91% (Table I). Significant responses were not obtained in two preparations.

Alcohol.—Injections of 0.2 ml. of alcohol (95%) produced a predominant decrease in outflow in four cases having a range of 4–10%. No change in volume outflow was recorded in two instances.

[1] Supplied through the courtesy of Ives-Cameron Laboratories, New York, N. Y.
[2] Supplied through the courtesy of Mead Johnson Research Center, Evansville, Ind.
[3] Supplied through the courtesy of Sterling-Winthrop Research Institute, Rensselaer, N. Y.

Propylene Glycol.—Propylene glycol caused a slight increase in outflow in two cases and a moderate decrease in two preparations.

DISCUSSION

The action of vasodilating agents is mediated through the activation of several different mechanisms, including (*a*) blood vessel musculature, (*b*) specific autonomic receptors and/or their chemical mediators, and (*c*) the central nervous system and its pathways. For example, guanethidine acts predominantly at the nerve-arteriole junction (11, 12) where it depletes norepinephrine in sympathetic nerve endings, thereby bringing about a vasodilating effect. Epinephrine, in small concentrations, produces a hypotensive effect which is mediated through stimulation of the inhibitory β-receptors (13). Papaverine (14), isosorbide (15), nitroglycerin (16), and cyclandelate (17) supposedly

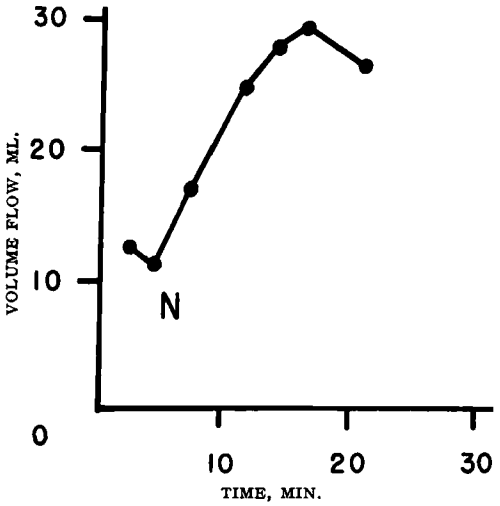

Fig. 3.—Effect of nitroglycerin spirit (2 mg.) on volume flow rate in vessels of the perfused human placenta.

inhibit or depress vessel musculature directly to elicit vasodilation. Isoxsuprine (18), in addition to its direct depressant action (papaverine-like) on vasculature, is thought to activate β-adrenergic receptors. The results of this investigation indicate that, generally, gross qualitative differences do not exist between the activity of the majority of vasodilators on placental vasculature and other circulatory beds.

The administration of cyclandelate to 15 different preparations produced either slight vasodilation or vasoconstriction. In four of these experiments the volume outflow increased above 10%, while in 11 preparations there was a decrease in volume outflow to 15%. In addition, the change in the volume outflow rate after cyclandelate administration in three other preparations was below 5%. Since responses obtained with cyclandelate were rather unpredictable and relatively slight, it is likely that placental vasculature does not possess the specific mechanism upon which this drug exerts its action. Furthermore, since consistent increases in volume outflow were obtained with papaverine and nitroglycerin, agents which affect vascular musculature directly, it follows that cyclandelate does not possess marked depressive musculotropic activity. Therefore, vasodilation that occurs in man after cyclandelate administration may be attributed mainly to some other pathway or mechanism that is not present in isolated placental preparations.

Isoxsuprine produced an increase in volume out-

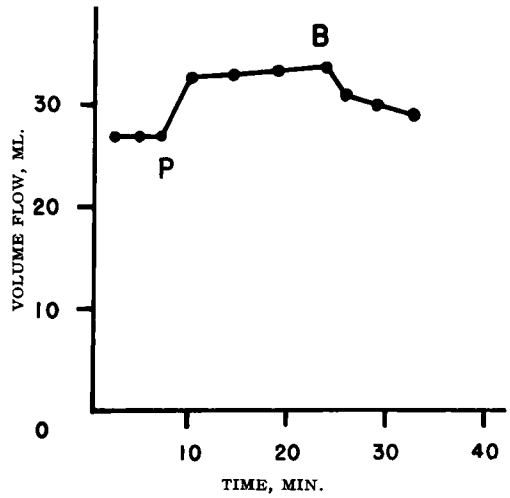

Fig. 4.—Effect of papaverine (5 mg.) and barium chloride (12.5 mg.) on volume flow rate in vessels of the perfused human placenta.

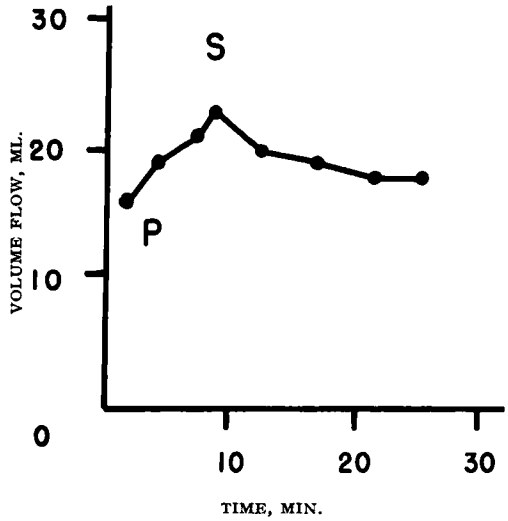

Fig. 5.—Effect of papaverine (5 mg.) and serotonin (50 mcg.) on volume flow rate in vessels of the perfused human placenta.

flow of over 5% in 10 preparations, a decrease of 5–11% in five, and either an increase or decrease of under 5% in 10. These varied responses would seem to indicate that, as with cyclandelate, placental vessels are devoid of a specific pathway upon which isoxsuprine acts. The activity of this agent on β-adrenergic receptors appears relatively slight since the degree and consistency of vasodilation are not comparable to that of isoproterenol which causes dilatation by activating these inhibitory receptors.

Vasodilation of the same magnitude as that produced by papaverine was never observed with isoxsuprine. Therefore, any papaverine-like activity that this agent may possess is relatively weak.

Treatment of placental preparations with isosorbide evoked responses that were qualitatively and quantitatively similar to those obtained from coronary artery studies in isolated hearts (19). These cardiac experiments showed that isosorbide produced an average increase in flow of 30–40% through coronary vessels, while in our investigation the average increment in volume outflow through placental vessels was 49%. Since both types of isolated preparations respond favorably to isosorbide administration, it appears that the effect of this agent depends mostly on its direct action on vascular musculature.

Isoproterenol brought about a sustained dilatation of vessels. However, this increase was not comparable to that occurring in response to alcoholic nitroglycerin or isosorbide. The mechanism of action of isoproterenol *in vivo* is due to stimulation of inhibitory β-receptors (12) and this logically could be the basis for its action on placental vessels.

Organic nitrates are known to be very active in causing dilatation of vessels; therefore, the increase in volume outflow produced in the placenta by nitroglycerin was anticipated. However, nitroglycerin spirit elicited a greater increase in flow than the aqueous preparation. It is possible that alcohol may have increased permeability in a manner to allow more effective cell contact, with the concomitant augmentation in response. This is not in accordance with the fact that the administration of 0.2 ml. of 95% alcohol did not cause dilatation of vessels but on the contrary caused appreciable vasoconstriction. The vasodilating action of alcoholic nitroglycerin was so powerful that subsequent administration of serotonin produced only very slight vasoconstriction.

Studies conducted by Eckenhoff and Hafkenschiel (20) indicate that papaverine is the most efficient coronary dilator. It should be noted that drugs which produce coronary dilation exert similar responses on placental vasculature. Likewise, in this experiment it was found that papaverine caused a prolonged dilation resulting in a marked increase in volume outflow. Furthermore, the action of papaverine was so intense that barium chloride and serotonin did not alter the dilation to any appreciable degree (Figs. 4 and 5).

The possibility exists, as indicated by von Euler (3), that the action of drugs on placental vasculature may correspond to their action on pulmonary vessels. An interesting aspect that should be explored is testing various agents on pulmonary and placental arteries and veins individually. It may be possible that these agents affect pulmonary and placental arteries and veins in a slightly different manner than other systemic arteries and veins which have a completely different oxygen content. Therefore, a correlation may exist between these two types of vessels and their reactions to drugs.

As indicated in a previous investigation (10), the possibility exists that fetal metabolism may be affected in part because of the action of drugs on placental vasculature. This is of significance if directly acting vasoconstricting agents are employed, since constriction of placental vessels may impair fetal tissue oxygenation. It should be kept in mind that in a pregnant patient with a previous history of hypotension the administration of vasoconstrictors may sensitize the placental vasculature and cause subsequent disturbance in fetal circulation. If placental vasoconstriction is suspected *in vivo*, certain of the vasodilators used in this investigation may be employed to counteract it.

Throughout this investigation, as in von Euler's study (3), a valid dose/response relationship does not appear to exist, as similar degrees of action occur with relatively small and large doses. This probably is because of the individual sensistivity of the placenta as well as to a probable lack of specific receptor sites.

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Production of Protein and Lipid by *Chlorella vulgaris* and *Chlorella pyrenoidosa*

By ROBERTSON PRATT and EVELYN JOHNSON

Cultures of *Chlorella vulgaris* reach the stationary phase of their growth cycle more slowly than cultures of *C. pyrenoidosa* when both are provided with conditions favorable for their growth. But after 3 weeks of incubation, the yields from the two organisms are nearly identical in terms of packed volume, dry weight, and cell population. There is only a relatively small difference in protein content—about 8 per cent in favor of *C. vulgaris*. There is a somewhat larger difference in lipid content, *C. pyrenoidosa* yielding about 11 to 12 per cent more lipid than *C. vulgaris*. Both species produce more lipid and relatively less protein with increasing age. Accumulation of lipid in excess of about 25 per cent of total dry weight is indicative of waning vigor in cultures of *Chlorella*, and a lipid content that equals or exceeds the protein content is indicative of extreme senescence. Both organisms grow poorly in a nitrogen-deficient medium but differ in their response to it. The yield from *C. vulgaris* is about 80 to 86 per cent of the yield from *C. pyrenoidosa* with respect to packed volume, dry weight, and per cent lipid. Only with respect to per cent protein are the two organisms the same. However, in absolute terms (mg./ml. of culture) *C. vulgaris* yields about 14 per cent less protein and nearly 30 per cent less lipid than *C. pyrenoidosa* in such a medium.

THE YIELD of conventional crop plants is markedly affected by the chemical composition and nutritive value of the substrate. But the ratio of protein/lipid/carbohydrate is not altered substantially. In contrast with this, it has been reported that the protein content of *Chlorella pyrenoidosa* can be varied from about 8 to 58% regularly (on one occasion a value of 88% was found) and that the lipid content can be varied from about 4.5% to more than 85% by simply altering the composition of the inorganic culture solution and properly controlling the incident light intensity, duration of culture, and other environmental factors (1). Carbohydrate concentration appears to be less amenable to alteration, the range being from about 5% to approximately 37%. All values were expressed as per cent of dry weight.

The concentration of nitrogen (or availability of nitrogen?) is reported to be a key factor. Spoehr and Milner (2) stated that high lipid values were never attained when the "residual fixed nitrogen concentration was greater than 0.001 molar" in the medium and that an initial nitrogen concentration of 0.025 *M* was sufficient to ensure that the plants would not reduce the level below 0.001 *M* and would not yield a high lipid value. Not all species of *Chlorella* respond uniformly in this respect. Such manipulation of the nitrogen concentration in the medium does not induce equivalent changes in the proportion of fatty acids produced by *C. vulgaris* (3).

The above observations, if regularly reproducible, would have potential practical import as well as theoretical interest. In the United States, Canada, Great Britain, Japan, and Israel there has been considerable interest in mass culture of *Chlorella* and other unicellular algae as potential future sources of food or food supplements. When the human race can no longer afford the inefficient luxury of the vast land areas and relatively long growth periods required to produce grain and meat and other conventional present-day foods, it may become necessary to resort to mass culture of unicellular algae to provide adequate sustenance for a large portion of the world population. It would be of considerable value to have knowledge of practical means for controlling the protein/lipid/carbohydrate ratio of the crop so that a custom-tailored diet, with respect to these major food components, could be produced. It will be important to know also how different species of algae compare with each other and with existing foods as sources of vitamins and of accessory growth factors. Some quantitative studies of vitamin content of algae and a few nutritional studies with animals have been reported (4–7), but no extensive or systematic evaluation has been published, especially with respect to changes that might occur with alteration of the basal composition of the harvest. The present paper deals with protein and lipid yields from *C. pyrenoidosa* and *C. vulgaris* cultured in media reputed to favor high protein and high lipid yields, respectively.

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EXPERIMENTAL

Materials and Methods

Organisms.—The strain of *C. vulgaris* employed

has been maintained in the laboratory since 1931 when it was isolated from a pond in Van Cortlandt Park, New York City. Identification was verified at that time by the late Professor Tracy Hazen of Columbia University. *C. pyrenoidosa* (an Emerson strain designated No. C-1-1-1 in the algal culture collection of the Kaiser Foundation Research Institue, Richmond, Cal.) was obtained through the courtesy of Dr. Mary Belle Allen of that institution.

Culture Conditions.—Inocula for experiments were always taken from 4-day stock cultures (inoculated from a long series of 4-day cultures) in liquid medium and were adjusted to provide an initial population of 100 cells/mm.^{3} (100,000/ml.). Stock cultures were illuminated continuously by a water-jacketed, 500-watt incandescent Mazda lamp. The total light intensity (as measured by a Weston photoelectric cell light meter, model 603) was 1,250 f.-c. at the position of the culture flasks. A mixture of 5% CO_2 and 95% air was bubbled through the cultures continuously. The temperature varied between 20 and 22°.

The standard solution for stock cultures and the reference base for experiments consisted of:

KNO_3	0.025 *M*
$MgSO_4 \cdot 7H_2O$	0.020 *M*
KH_2PO_4	0.018 *M*
$FeSO_4 \cdot 7H_2O$	5×10^{-5} *M*
$K_3C_6H_5O_7 \cdot H_2O$	5×10^{-5} *M*
Zn(as $ZnSO_4 \cdot 7\ H_2O$)	0.400 p.p.m.
Cu(as $CuSO_4 \cdot 5H_2O$)	0.004 p.p.m.
Mn(as $MnSO_4 \cdot 4H_2O$)	0.400 p.p.m.
B(as H_3BO_3)	0.020 p.p.m.

A solution containing the three major salts was sterilized in the autoclave (15 minutes at 18-lbs. pressure). The other salts were incorporated in a single solution which was sterilized by filtration and then added aseptically to complete the formulation. The pH of the uninoculated medium is 4.5 to 4.6. As the culture develops, the pH rises to a value of 7.0 to 7.5 in 2 weeks and tends to remain at that level, even with prolonged incubation.

Columnar Pyrex culture vessels (60.5 cm. tall × 43 cm. diam. and each containing 500 ml. of culture medium) were arranged in a circle around a central, water-cooled incandescent light bank consisting of four clear, 60-watt Lumiline show case lamps, which provided 600 f.-c. continuously at the culture vessels. The temperature was 20.5 ± 0.5°.

A 5% CO_2–95% air mixture was introduced continuously through a sintered-glass sparger at the bottom of each vessel and bubbled up through the cultures in a finely dispersed stream which served the dual purpose of providing CO_2 for photosynthesis and retarding settling of the cells. Even so, daily shaking was necessary to maintain a uniform suspension.

Under exceptional circumstances, the cultures developed a population density of about half a million cells/mm.^{3} (actual maximum count = 506 million/ml.) after 3 weeks of growth; but the usual plateau level attained at that time was about 330 to 340 million cells/ml.

The C-2 formulation of Spoehr and Milner (2), henceforth designated SM, was selected as the high-lipid medium. It contains

$(NH_4)H_2PO_4$	0.000825 *M*
$(NH_4)_2HPO_4$	0.000715 *M*
KCl	0.03 *M*
$MgSO_4 \cdot 7H_2O$	0.01 *M*
KH_2PO_4	0.01 *M*
[Fe]	5×10^{-6} *M*

No trace elements (other than iron) were added by the original formulators, since they used "local tap water" which had a "fairly high mineral content," and they found that "the yields of *Chlorella* indicated that the water was adequate for providing micro-elements." However, media for the present experiments were prepared with distilled water. Therefore, micronutrients were added in the same concentration as for the standard medium. Thus, the concentration of iron added in our experiments was ten times that added by Spoehr and Milner. Since the iron content of the tap water they employed is not known, the precise difference in concentration of iron in the two sets of experiments cannot be stated. But it is reasonable to assume that it was at least several-fold higher in our experiments than in theirs. The initial pH of the SM medium before inoculation is 5.9. This value drops rapidly as the organisms begin to grow and soon stabilizes at about pH 3.5. This is consistent with the earlier observations of Pratt and Fong (8).

Analytical Methods

Upon harvest, the cell suspensions were centrifuged and the cell mass was resuspended and washed in distilled water. In harvesting cultures that had nitrogen supplied as ammonium salts, the cells were washed repeatedly until the supernatant gave a negative reaction with Nessler's reagent.

After the final washing, the cells were resuspended in distilled water (0.1 the original harvested volume). Aliquots of this tenfold concentrated suspension were used for determination of packed volume and dry weight by conventional methods. Cell counts were determined with a haemocytometer from the original cell suspension before centrifuging.

Protein was determined by the standard Kjeldahl micro-method (range 10 to 40 mcg. nitrogen) as outlined by Umbreit, *et al.* (9), with modifications adapted from Johnson (10) and Schmidt (11).

Lipids were determined by a procedure adapted from Milner (12), using a paste of freshly harvested cells [100–500 mg. (dry wt.)]. The cell mass was extracted twice with methanol under reflux; then extracted by refluxing three times each with ethyl ether and methanol, alternately, over a period of 2 days. The several fractions were filtered, pooled, and evaporated to dryness. The dry residue was re-extracted with ethyl ether three times to eliminate methanol-soluble sugars. The final ether extract was evaporated to dryness and brought to constant weight in a vacuum desiccator. A fresh 30-ml. portion of solvent was used for each extraction throughout the procedure.

Precision of Methods and Reproducibility of Data

In work of the type reported below it is important to indicate the degree of reproducibility of the data (adequacy of sampling) and the precision of the analytic procedures employed to obtain them (methodology). Among factors affecting adequacy

of sampling we include all the numerous physical factors and manipulative procedures to which the cultures are subjected.

The coefficient of variability (standard deviation expressed as a percentage of the mean $=v$) was chosen as the most appropriate statistical indicator because it relates not only to the reproducibility of one set of data, but also indicates the reliability and reproducibility of several kinds of data. In general, variabilities of two or more distributions, regardless of their units of measurement, may be considered to be related to each other as the corresponding values of v. The procedures upon which the conclusions presented below are based were determinations of dry weight, nitrogen, and lipid. Run on aliquots of a given sample, such determinations routinely yielded highly reproducible values. The critical factors affecting reproducibility of data are in the cultures. Inherent biologic variation cannot be ruled out of any work with living organisms, but strains of *Chlorella* that have been under long laboratory cultivation appear to be quite stable. We believe that the major sources of variability are in certain physical culture conditions which cannot reasonably be controlled with absolute precision and the extensive but necessary manipulation of the cultures from the time of set-up through the time of harvest and preparation of the cells for analysis. In any given run of replicate cultures, some variation occurs. Values of v calculated for duplicate to quadruplicate samples from each of ten cultures were within the following limits: mg. dry wt./ml., 0 to 1.3%; mcg. nitrogen/ml., 0.5 to 5.1%; mg. lipid/ml., 0 to 5.5%.

When averaged data from several series of cultures in different experiments conducted for different lengths of time are compared, larger coefficients of variability are to be expected. Values of v for the data tabulated under *Results* were within the following limits: mg. dry wt./ml. 14 to 14.2%; protein as per cent of dry weight, 6.7 to 8.6%; and lipid as per cent of dry weight, 7.2 to 18%. Packed volume and cell counts are recorded in some of the following tables primarily to round out the picture; they did not form the basis for the specific conclusions reached. The coefficients of variability for these potential indicators of growth in all of the experiments were within the following limits: packed volume, 9.6 to 23.4%; cell count 17.1 to 17.5%.

RESULTS

Averaged data from 12 replicated experiments are summarized in Table I. The comparative data in Table II parts A and B, (based on the figures in Table I) indicate, respectively, the relative value of the standard and the "high-lipid-inducing" media for each organism and the relative performance of the two organisms in each medium.

The tables show clearly the superiority of the standard over the SM medium for growth of both species of *Chlorella*, as indicated by packed volume, dry weight, and protein content. Only with respect to lipid do the cells produce a lower percentage yield in the standard medium than in SM (Table I and Table II, part A, columns *1–4*). The tables also show differences in the responses of the two organisms to the different media, as discussed below.

Packed Volume and Dry Weight.—After 2 weeks, protein content, dry weight, and packed volume of *C. vulgaris* cultured in standard medium are about three to four times those produced in SM medium (Table II, part A, column *1*). After another week, the differences are approximately 3- to 5.5-fold in favor of the standard (Table II, part A, column *2*). A similar trend, differing only in detail, occurs in cultures of *C. pyrenoidosa* (Table II, part A, columns *3* and *4*). During the third week of incubation, the increase in relative dry weight in the standard *versus* the SM medium is less for *C. pyrenoidosa* than for *C. vulgaris;* during the same period the relative values for packed volume and protein decrease in cultures of *C. pyrenoidosa*, while they increase in cultures of *C. vulgaris*. In other words, the superiority of the standard medium over the "high lipid" medium for supporting *C. vulgaris* is more apparent after 3 weeks than after 2 weeks, but the same is not true for cultures of *C. pyrenoidosa*. This can be ascribed, in part, to more rapid attainment of "maturity" by cultures of the latter organism than of the former when both are in standard medium.

For example, the yield in terms of packed volume and dry weight is essentially the same from both organisms at 3 weeks in standard medium (Table I, columns *2* and *6*; Table II, part B, column *6*). But *C. pyrenoidosa* produces about 90% of its packed volume and about 80% of its dry weight in the first 2 weeks of growth (compare columns *5* and *6*, Table I), while the corresponding figures for *C. vulgaris* are 70 and 64% (compare columns *1* and *2*, Table I). Stated another way, *C. vulgaris* still has 30 to 35% of its growth to produce in the third week under the conditions described, but *C. pyrenoidosa* has only 10 to 20% of its yield remaining to be produced. Because of this difference in the growth pattern of the two organisms in the standard medium, and since neither of them makes a substantial gain in packed volume or dry weight during the third week in SM medium (Table I, columns *3*, *4*, *7*, and *8*),

TABLE I.—YIELDS FROM *C. vulgaris* AND *C. pyrenoidosa* IN STANDARD (STD.) AND IN "HIGH-LIPID-INDUCING" (SM) MEDIA (LIGHT = 600 F.-C.)

	C. vulgaris				*C. pyrenoidosa*			
	Std. Medium		SM Medium		Std. Medium		SM Medium	
	13–14 days (*1*)	20–21 days (*2*)	13–14 days (*3*)	20–21 days (*4*)	13–14 days (*5*)	20–21 days (*6*)	13–14 days (*7*)	20–21 days (*8*)
Packed vol. (ml./65 ml. of culture)	0.848	1.200	0.198	0.215	1.100	1.186	0.24	0.27
Dry weight (mg./ml. of culture)	2.623	4.134	0.842	0.896	3.316	4.182	1.017	1.039
Cell count (per mm.^{3})	213,600	338,857	n.c.[a]	n.c.[a]	183,042	332,750	n.c.[a]	n.c.[a]
Protein (% dry wt.)	52.7	48.58	17.7	15.6	56.46	44.83	16.2	15.6
Lipid (% dry wt.)	21.3	20.45	30.7	37.7	21.8	23.1	37.9	44.8

[a] n.c. = Cells not countable because of adhesion and excessive clumping.

TABLE II.—A, RELATIVE VALUE OF STANDARD (STD.) AND OF "HIGH-LIPID-INDUCING" (SM) MEDIA FOR SUPPORTING *C. vulgaris* AND *C. pyrenoidosa*. B, YIELDS OF *C. vulgaris* WITH RESPECT TO YIELDS OF *C. pyrenoidosa* IN STANDARD (STD.) AND IN "HIGH-LIPID-INDUCING" (SM) MEDIA

	A				B			
	C. vulgaris		*C. pyrenoidosa*		Std. Medium		High-Lipid-Inducing Medium	
	Std./SM[a]		Std./SM[a]		*vulgaris/pyrenoidosa*[b]		*vulgaris/pyrenoidosa*[b]	
	13–14 days (1)	20–21 days (2)	13–14 days (3)	20–21 days (4)	13–14 days (5)	20–21 days (6)	13–14 days (7)	20–21 days (8)
Packed vol.	4.28	5.58	4.58	4.40	0.77	1.01	0.82	0.80
Dry wt.	3.12	4.61	3.26	4.02	0.79	0.99	0.82	0.86
Cell count	n.d.[c]	n.d.[c]	n.d.[c]	n.d.[c]	1.17	1.02	n.d.[c]	n.d.[c]
Protein[d]	2.97	3.11	3.48	2.87	0.93	1.08	1.09	1.00
Lipid[e]	0.69	0.54	0.57	0.52	0.98	0.89	0.81	0.84

[a] Std./SM = Yield in standard medium ÷ yield in SM medium. [b] *vulgaris/pyrenoidosa* = Yield from *vulgaris* ÷ yield from *pyrenoidosa*. [c] n.d. = Not determined because cells in SM medium too clumped to permit accurate counting. [d] Calculated from protein expressed as per cent of dry weight. [e] Calculated from lipid expressed as per cent of dry weight.

the disparity between the two media after 3 weeks will be greater for *C. vulgaris* than for *C. pyrenoidosa*. (See Table II, part A, columns *2* and *4*).

Protein Content.—The protein content (expressed as per cent of dry weight of the cells) of both organisms decreases during the third week, regardless of the medium (Table I). However, when the magnitude of the increase in dry weight during that period is taken into consideration, it is found that in terms of absolute yield of protein *C. vulgaris* shows a substantial gain in the standard medium (1.38 mg./ml. of culture at 2 weeks *versus* 2.01 mg./ml. at 3 weeks), whereas cultures of *C. pyrenoidosa* remain virtually static in this respect (1.872 *versus* 1.874), as do both organisms in SM medium (*C. vulgaris* 0.149 *versus* 0.140 at 2 weeks and 3 weeks, respectively: *C. pyrenoidosa* 0.164 *versus* 0.162).

The protein content of *C. vulgaris* in standard medium at 3 weeks is only 3.75% higher than that of *C. pyrenoidosa* when expressed as per cent of dry weight (Table I) but in relative terms (Table II, part B, column *6*) and in terms of absolute yields (2.01 *versus* 1.87 mg./ml. of culture) is about 8% higher. This is just the reverse of the situation at 2 weeks (Table I, column *5* minus column *1;* and Table II, part B, column *5*) and may be taken as further evidence that cultures of *C. vulgaris* attain full productivity more slowly than those of *C. pyrenoidosa* in the standard medium.

Lipid.—*C. pyrenoidosa* has a higher lipid content than *C. vulgaris* at 2 weeks and at 3 weeks, regardless of the medium employed and of whether values are expressed as per cent of dry weight or in absolute terms of mg. of lipid per ml. of culture; both organisms produce more lipid, relative to their dry weight, in SM medium than in the standard (Table III). The percentage lipid content of both organisms grown in SM medium is approximately two to three times their protein content (Table I).

Chlorella cells in SM medium are distinctly abnormal in comparison with those in standard medium. Normal, healthy cultures are a clear, pure green, and the cells are separate, discrete and, when the cultures are shaken, readily form a uniform suspension. Although there is some tendency for settling out, a large proportion of the cells remain in suspension. The cells do not clump, and haemocytometer counts of cell population can be made satisfactorily. Viewed under the microscope, the cells are smooth and rounded; they present a clear, sharp outline.

In contrast to the above, cultures in SM medium

TABLE III.—LIPID CONTENT OF *Chlorella* CULTURES IN STANDARD (STD.) AND IN "HIGH-LIPID-INDUCING" (SM) MEDIA (LIGHT = 600 F.-C.)

Lipid Content	Std. Medium 13–14 days	Std. Medium 20–21 days	SM Medium 13–14 days	SM Medium 20–21 days
	C. vulgaris			
As % of dry weight	21.3	20.45	30.7	37.7
Mg./ml. of culture	0.559	0.845	0.258	0.338
	C. pyrenoidosa			
As % of dry weight	21.8	23.1	37.9	44.8
Mg./ml. of culture	0.723	0.966	0.385	0.465

acquire a distinctly yellowish cast after 5 to 6 days. About 10 days after inoculation cells no longer form a uniform suspension when the cultures are shaken. They are clumped in irregular aggregates, probably containing from a few thousand to several million cells, and the aggregates cannot be separated sufficiently to permit accurate or reproducible counts using the haemocytometer method. The clumps do not long remain in suspension: either they settle out quickly or rise with the gas stream to the surface of the culture where most of them adhere to the sparger inlet tube or to the side of the culture vessel. Many cultures (especially of *C. vulgaris*) soon become virtually water-clear except for a short space near the bottom of the vessel and for the cells adhering to the sparger tube or culture wall at the surface of the medium. Microscopic examination shows the margins of the cells to be wrinkled and convoluted. The cells are several times larger than cells of comparable age in standard medium and each cell appears to contain many smaller cells, often containing still smaller units, as though division had not been completed or, if completed, had not been followed by release of the daughter cells. The pattern is reminiscent of that seen in aged cultures that have been in standard medium for many weeks. It is obvious that the cultures in SM medium are unhealthy, if not actually moribund.

Others also have commented on the altered appearance of cultures in high-lipid-inducing media, noting their yellowish color, changes in the character of the cells, and suggesting that "cells with a high lipid content carry on photosynthesis with a chlorophyll content 1/500 to 1/2000 that of cells with low lipid content" (2). It has been stressed

TABLE IV.—A, DRY WEIGHT, PERCENTAGE PROTEIN, AND LIPID YIELDS FROM *C. vulgaris* AND *C. pyrenoidosa* EXPOSED TO HIGH LIGHT INTENSITY (1250 F.-C.). B, YIELD OF PROTEIN AND LIPID EXPRESSED IN ABSOLUTE TERMS (MG./ML. OF CULTURE)

	C. vulgaris				*C. pyrenoidosa*			
	Std. Medium		SM Medium		Std. Medium		SM Medium	
	2 wks. (*1*)	6 wks. (*2*)	2 wks. (*3*)	6 wks. (*4*)	2 wks. (*5*)	6 wks. (*6*)	2 wks. (*7*)	6 wks. (*8*)
A								
Dry weight (mg./ml. of culture)	2.373	5.009	1.173	1.216	2.276	5.573	1.051	1.271
Protein (% dry wt.)	50.5	32.2	13.6	12.5	56.6	27.8	15.2	10.5
Lipid (% dry wt.)	7.5	19.7	16.8	29.4	13.3	26.2	24.3	43.0
B								
Protein (mg./ml. of culture)	1.198	1.613	0.160	0.152	1.276	1.549	0.160	0.133
Lipid (mg./ml. of culture)	0.178	0.987	0.197	0.358	0.303	1.460	0.255	0.547

that to induce development of high-lipid-cells a long growth period and high light intensity are required in addition to low nitrogen concentration in the medium (2). A precise quantitative definition of high light intensity was not given, but calculations based on informed estimates suggested that it was approximately comparable to the 600 f.-c. level adopted for the present work. However, since the maximum lipid value found in the present investigation was 20 to 30% lower than the values reported by the previous workers for a similar medium, it seemed desirable to extend the culture period and to increase the incident light.

Replicate cultures of *C. vulgaris* and of *C. pyrenoidosa* were started in standard and in SM media and were exposed to 1250 f.-c. The column culture vessels were replaced by Fernbach flasks illuminated from below by a water-screened bank of thirty 100-watt Mazda lamps and by Florence flasks placed around a central, water-jacketed 500-watt Mazda lamp. These conditions more nearly simulated those provided by the earlier investigators. The conventional 5% CO_2 in air was provided. The temperature was 22.5 ± 2°.

All cultures in SM medium became progressively more unhealthy, flocculent, and "scummy" looking after the second week. It was mentioned above that (column) cultures with 600 f.-c. illumination acquire a yellowish cast after 5 to 7 days. In the (flask) cultures provided with higher light intensity, the cells become distinctly yellow—so much so that the pellet remaining after cultures are spun down in the centrifuge is clearly yellow with no trace of green. Microscopic examination revealed no contamination. Results from the cultures harvested after 2 weeks and 6 weeks are shown in Table IV.

The dry weight of *C. pyrenoidosa* in SM medium increased only 20% between the end of the second and sixth weeks (Table IV, part A, column *8* ÷ column *7*). In standard medium it increases approximately 145% in the same period (column *6* ÷ column *5*). In both media, the lipid content of the organisms increases considerably during this period, regardless of whether the yields are expressed in terms of per cent of dry weight (Table IV part A, columns *5–8*) or in terms of absolute values (Table IV, part B). However, in terms of the actual amount of lipid formed between the second and sixth weeks, the increase was almost four times greater in the standard medium—1.157 *versus* 0.292 mg./ml. of culture. It was greater on a relative basis also—4.8-fold *versus* 2.1-fold (compare Table IV, part B, column *6* ÷ column *5* with column *8* ÷ column *7*).

C. vulgaris is far less prone than *C. pyrenoidosa* to produce lipid in either medium (Table IV, parts A and B) but, even so, with *C. vulgaris* also the absolute increase in lipid was greater in the standard medium than in SM—0.809 *versus* 0.161 mg./ml. of culture (compare Table IV, part B, column *2* minus column *1* and column *4* minus column *3*). The ratio of the increases in the two media is approximately 5 to 1 in favor of the standard, a figure remarkably close to the 4.8 to 1 ratio found with *C. pyrenoidosa*.

Both organisms, regardless of the medium, give substantially lower protein yields (as per cent of dry weight) after 6 weeks than after 2 weeks (Table IV, part A). However, in terms of the actual amount of protein recoverable from the cells, both organisms make a slight gain during the extended growth period in the standard medium (Table IV, part B). But in SM medium, despite the smaller percentage decreases (Table IV, part A), there is an actual loss of protein (Table IV, part B). This emphasizes the necessity of considering absolute as well as relative yields in experiments of this kind.

The increase in protein, expressed in absolute terms of mg./ml. of culture, between the end of the second and sixth weeks in standard medium (Table IV, part B), accounted for approximately 16% of the total increase in dry weight (Table IV, part A) of *C. vulgaris* and about 8% of the total increase in dry weight of *C. pyrenoidosa*. But increase in lipid calculated in the same way accounted for 31% of the increase in total dry weight of *C. vulgaris* and about 35% of the increase in total dry weight of *C. pyrenoidosa*. Stated another way, the lipid yield from *C. vulgaris* in standard medium doubled with respect to protein yield between the end of the second and sixth weeks and the yield from *C. pyrenoidosa* quadrupled.

DISCUSSION

The experiments described above confirm the observation that *Chlorella* has a higher lipid and lower protein content (both expressed as per cent of dry weight) when cultured in a nitrogen-deficient medium than when provided with an adequate amount and form of nitrogen. However, the experiments show clearly that, important as percentages may be in terms of fundamental biology or biochemistry, values expressed in percentages provide only a partial and sometimes misleading picture. Two examples will be cited. Others have been pointed out

above, and still others can be found by making appropriate calculations from the tabulated data.

Percentage data in Table I indicate less than 4% difference in per cent protein of *C. pyrenoidosa* and of *C. vulgaris* after 2 weeks in column cultures with standard medium and 600 f.-c. illumination. But in terms of actual amount of protein (mg./ml. of culture), the yield from the former was about 35% greater than from the latter. The discrepancies become even greater when performance in different media is considered. After 6 weeks in SM medium in flask cultures with twice as much light, the lipid content of *C. pyrenoidosa* was 43% *versus* 26.2% for the same organism in standard medium—a difference of 16.8% in favor of SM medium. But the actual amount of lipid present in the cultures in standard medium was more than 2.5 times the amount present in cultures reared in the nitrogen-deficient medium, and, as was pointed out above, the actual production of lipid during the last 4 weeks of cultivation was nearly five times as great in the standard as in the nitrogen-deficient medium. It is evident that if one wished to produce fats microbiologically using *Chlorella* as the biosynthetic mediator, much greater yields could be obtained in less time from healthy cells cultured in the standard medium than from the unhealthy cells produced in the SM medium—the higher percentage yields from the latter notwithstanding. It should be pointed out that the composition of *Chlorella* lipids may vary depending on the medium in which the cells develop. Milner (12) noted that the fatty acid content of lipid from high-lipid cells was about three times that of the lipid from low-lipid cells but that the unsaponifiable fraction of the latter was more than three and one-half times greater than that of the former. Even so, in view of the very poor development in SM medium, it is likely that normal cells in standard medium would provide higher absolute yields of any of the lipid fractions than would cells produced in the inferior medium.

Although confirming that *Chlorella* produced in SM medium has a higher percentage of total lipid than those produced in the standard medium, we have been unable to obtain percentage values as high as those reported by Spoehr and Milner (2). An unequivocal explanation for the apparent discrepancy cannot be offered because the experimental conditions used by them cannot be duplicated due to the lack of specific details with respect to light intensity, concentration of trace elements in the medium, and other important factors. It is not possible to obtain a culture of *C. pyrenoidosa* that can be stated authoritatively to be the same Emerson strain as the one with which they worked. It is not clearly stated in the Spoehr and Milner paper that most of their cultures were "aerated" with a carbon dioxide–nitrogen mixture, and we learned this inadvertently only after the present work (employing the conventional carbon dioxide–air mixture, was completed. Whether substitution of nitrogen for air in the "aeration" mixture has a significant effect on the formation of lipid and protein and on the ratio of one to the other is under investigation.

It has been stated that "a culture with any previously obtained R-value [which may be taken as an indicator of per cent lipid] could be grown again at will" (2). In the light of our experiments, we interpret that statement to mean that a given strain of *Chlorella* tends to be genetically stable and remarkably uniform in its response to alterations in the environment. One such response to deficient nitrogen supply is an increased percentage of lipid in the cells—a phenomenon that is a concomitant of senescence and waning vigor and occurrence of which is accelerated in the unfavorable high-lipid-inducing medium.

SUMMARY

Two species of unicellular green algae, *C. vulgaris* and *C. pyrenoidosa*, have been compared with respect to production of protein and of lipid in two different media—one reputed to favor development of high protein content and the other to favor high yields of lipid. The two species were found to differ in their rates of achieving full development and in their tendency to produce protein and lipid.

The standard medium supports healthy, vigorous growth of both species. In this medium *C. pyrenoidosa* attains full development more rapidly than *C. vulgaris*, but the latter has a higher protein content, both in terms of percentage of dry weight and in terms of absolute amount. *C. pyrenoidosa* yields more lipid, both in terms of percentage and of absolute amount. The yield of protein, as per cent of dry weight but not in absolute terms of mg./ml., decreases as cultures of either organism approach full development. Concurrently, the lipid content increases in both species.

When the source of nitrogen is sufficiently limited (situation reputed to favor development of high lipid content) the cultures soon become yellow and sickly. Under these conditions, the lipid content of both organisms (expressed as per cent of dry weight) is approximately two or more times that found in normal healthy cultures of the same age. But the absolute amount of lipid present at full development is markedly less than in normal cultures because of the very much lower dry weight produced under these conditions.

The lipid content of *C. pyrenoidosa* was consistently higher than that of *C. vulgaris* regardless of the culture medium used and whether the content is expressed as percentage of dry weight or in terms of absolute yield.

All the experiments indicated that substantial increase in the proportion of total dry weight accounted for as lipid accompanies aging of the cultures of both species. When the lipid content, expressed as percentage of total dry weight, closely approaches, equals, or exceeds the protein content, the cells are extremely senescent, if not actually moribund.

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Thalictrum Alkaloids I

Thalicarpine, A New Hypotensive Alkaloid from *Thalictrum dasycarpum*

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A preliminary study of the roots of *Thalictrum dasycarpum* Fisch. and Lall from Wisconsin yielded magnoflorine and a new alkaloid, thalicarpine. Evidence is presented for assignment to thalicarpine of the empirical formula $C_{41}H_{48}O_8N_2$. Thalicarpine was evaluated in a variety of pharmacologic procedures and found to possess a modicum of biological activity.

THALICTRUM TOURN. EX LINN. (*Ranunculaceae*), commonly known as "meadow rue," is a widely distributed genus. Many medicinal uses of *Thalictrum* species in folk remedies have been recorded. *T. foliosum* DC. is found throughout the Himalayas and taken as a tonic, aperient, purgative, diuretic, febrifuge, a remedy for atonic dyspepsia, and used as an application for ophthalmia (1). *T. thunbergii* DC. is used in Japan as a home remedy against stomach ache and diarrhea (2). *T. collinum* Walbr., *T. angustifolium* L., and *T. silvaticum* Koch. have been used in Ukrainian folk medicine as diuretics (3). *T. fendleri* Engelm. was prepared by the Indians of Nevada as a tea to cure gonorrhea; a decoction of the root was used against colds (4). *T. minus* L. is used in south Africa to treat fevers (5). Intravenous injection of the hydrochlorides of the extract of the total alkaloids from *T. minus* L. has recently been shown to exert an effect on the blood pressure and pulse of frogs, cats, and dogs (6).

In the course of a continuing screening program for alkaloid-bearing plants, the roots of *Thalictrum dasycarpum* Fisch. and Lall[1] from Wisconsin were found to afford substantial yields of alkaloids. Pharmacological evaluation of the nonquaternary alkaloid fraction demonstrated potent hypotensive activity in the anesthetized cat after intravenous administration.[2] The present report describes a preliminary study of the alkaloids of *T. dasycarpum* and the isolation and characterization of thalicarpine, a new hypotensive tertiary base. While our work was in progress, the isolation from *T. dasycarpum* of the quaternary alkaloids magnoflorine and berberine was reported (7). However, no work on the nonquaternary alkaloids has appeared to date.

Coarsely ground plant was extracted successively with methanol and 1.5% triethylamine in methanol. The extracts were processed for alkaloid content by the procedure summarized in Fig. 1, whereby an 0.35% yield of crude alkaloids was obtained. Fraction E, the quaternary alkaloid fraction, yielded magnoflorine as the only isolatable component. Study of the nonquaternary phenolic alkaloid Fractions B and D is in progress and will be reported in due course.

The nonquaternary nonphenolic alkaloid Fractions A and C were combined on the basis of their paper chromatographic patterns. Chromatographic fractionation led to isolation of the major alkaloid of the plant (0.07% of the dried roots). The compound showed m.p. 160–161°, $[\alpha]_D^{25}$ + 133° (methanol); + 89° (chloroform), and ultraviolet, infrared, and N.M.R. spectral characteristics which indicated that the material is a new compound. The name *thalicarpine*, reflecting the botanical origin, is proposed for the alkaloid.

The molecular formula $C_{41}H_{48}O_8N_2$ was assigned for thalicarpine on the basis of elemental analysis and molecular weight determination by nonaqueous titration. Analysis showed the presence of seven O-methyl groups and two N-methyl groups. The N.M.R. spectrum in deuterated chloroform solution supports the formula, showing six N-methyl protons, 21 O-methyl protons, 14 aliphatic protons, and seven aromatic protons. The infrared spectrum indicates the presence of aromatic rings and aromatic O-methyl groups, but absence of hydroxyl, carbonyl, and isolated double-bonds.

A sequence of derivatives was prepared to seek confirmation of the empirical formula as well as to provide conversion products useful for structure elucidation. Treatment of thalicarpine with methyl iodide afforded a product which resisted all attempts at crystallization. However, treatment of the amorphous methiodide with alkali yielded a Hofmann methine which was converted to a crystalline methiodide. Analysis afforded results which support a $C_{45}H_{58}O_8N_2I_2$ formula for the Hofmann

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[1] Air dried roots, collected in southern Wisconsin during the summers of 1958, 1959, and 1960. We thank Professor H. H. Iltis, University of Wisconsin, for confirming the identity of the plant. A voucher specimen is deposited in the University of Wisconsin Herbarium.

[2] The authors thank Mr. Edward Macko, Smith Kline and French Laboratories, Philadelphia, Pa., for the pharmacological results reported herein.

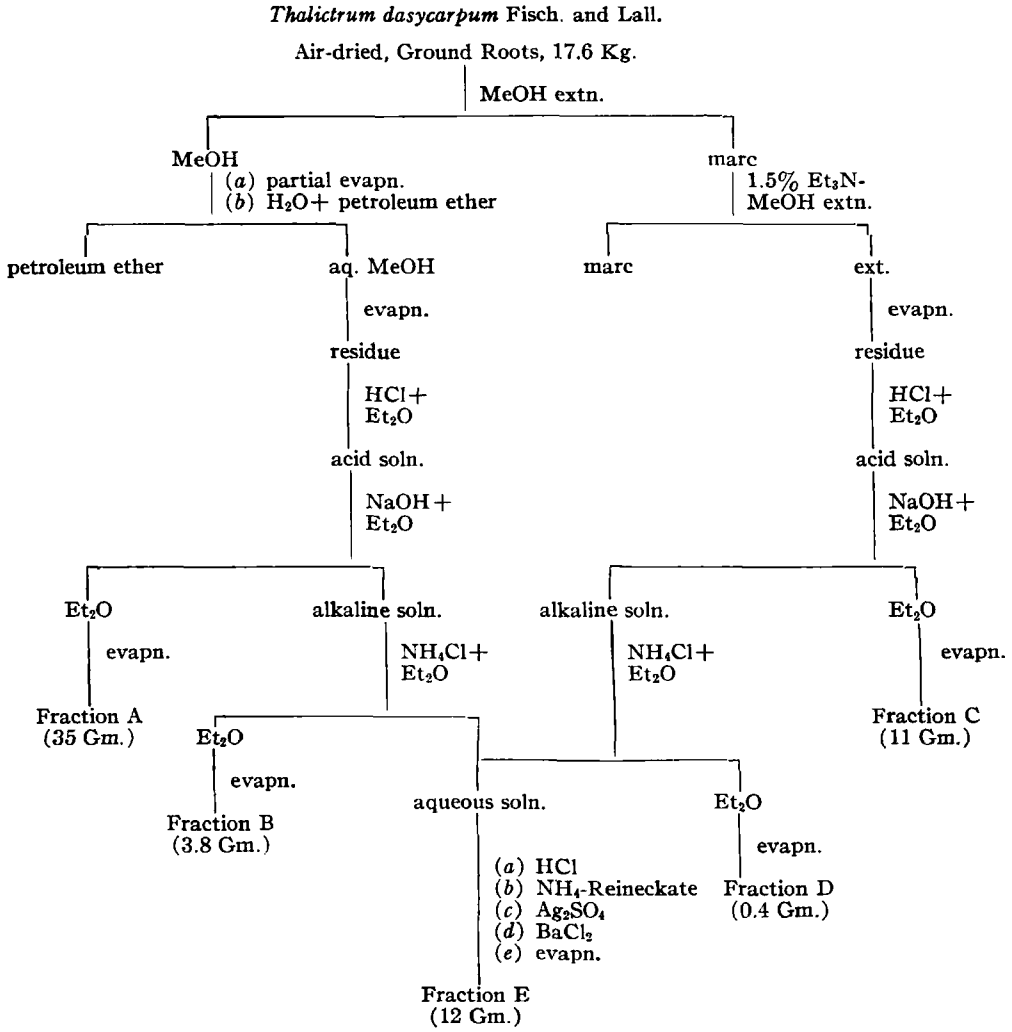

Fig. 1.—Flow sheet for separation of alkaloids of *Thalictrum dasycarpum* Fisch. and Lall.

methine methiodide. A second Hofmann degradation yielded a des-N-methine, which afforded analytical results indicative of a $C_{39}H_{38}O_8$ empirical formula. The formulas of both methine derivatives support the $C_{41}H_{48}O_8N_2$ formula for thalicarpine. Further structural studies are in progress and will be reported at a later date.

EXPERIMENTAL

Melting points have been corrected for stem exposure. Values of $[\alpha]_D$ have been approximated to the nearest degree. Infrared spectra were determined on a Beckmann IR-5A infrared spectrophotometer. Ultraviolet spectra were determined in methanol on a Cary recording spectrophotometer. The N.M.R. spectra were taken on a Varian A-60 N.M.R. spectrometer. Paper chromatography was conducted by the descending technique on Whatman No. 4 paper pretreated with buffer at pH 3.5.

Extraction of Alkaloids from Thalictrum dasycarpum. Separation into Main Fractions

Coarsely ground *T. dasycarpum* (air dried roots, 17.6 Kg., from middle and southern Wisconsin) was continuously extracted with methanol in a Soxhlet-type extractor. The extraction was continued with a fresh charge of solvent at the end of 3 days. When the extraction was stopped after a total of 10 days, the extract returning to the pot yielded a residue which did not give a positive Mayer's test upon evaporation. The methanol extract was concentrated under reduced pressure to a dark brown semisolid concentrate. The 1 L. concentrate was diluted with 2 L. of water and extracted with 3 L. of petroleum ether (Skellysolve B, b.p. 60–68°) to remove fat. The aqueous methanolic solution was further evaporated to remove most of the methanol, then triturated with 1.5% hydrochloric acid. After rejecting acid insoluble material, the acidic solution was washed with 6 L. of ether, made alkaline with sodium hydroxide, and extracted with 6 L. of ether. The ether extract was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure to yield a glass-like alkaloidal residue (Fraction A, 35 Gm.; see Fig. 1). The alkaline solution re-

maining from ether extraction was made more weakly basic to pH 8.5 by adding ammonium chloride and extracted with 6 L. of ether. The ether extract was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure to yield a semisolid alkaloidal residue (Fraction B, 3.8 Gm.). The aqueous solution remaining from ether extraction was reserved for study of the quaternary alkaloids.

Next, the marc remaining from the methanol extraction was extracted continuously with 1.5% trimethylamine in methanol for 12 days. When extraction was stopped, evaporation of the extract returning to the pot yielded a residue which did not give a positive test with Mayer's reagent. The trimethylamine-methanol extract was evaporated to dryness, and the residue was triturated with 1.5% hydrochloric acid (1 L.). After rejecting acid-insoluble material, the acidic solution was washed with ether, made alkaline with sodium hydroxide, and extracted with 2 L. of ether. The ether extract was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure to yield 11 Gm. of alkaloidal residue (Fraction C). The basicity of the aqueous solution remaining from ether extraction was adjusted to pH 8.5 by adding ammonium chloride. The alkaline solution was then extracted with 2 L. of ether. The ether extract was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness to yield a semisolid alkaloidal residue (Fraction D, 0.4 Gm.). The aqueous alkaline solution was combined with the corresponding aqueous solution from the methanol extract. The combined solution was re-acidified with concentrated hydrochloric acid and treated with a saturated Reinecke salt solution. The dried precipitate was dissolved in acetone, the solution was clarified by filtration, and treated with a saturated aqueous silver sulfate solution to complete precipitation. Silver Reineckate was removed by filtration; the filtrate was treated with a barium chloride solution to complete precipitation. After removal of barium sulfate by centrifugation, the supernatant solution was evaporated to dryness under reduced pressure to yield 12 Gm. of quaternary chloride mixture (Fraction E).

Isolation of Alkaloids

Fractions A and C, constituting the nonquaternary nonphenolic alkaloids, were combined on the basis of the similarity of their paper chromatographic patterns. Similarly, Fractions B and D, constituting the nonquaternary phenolic alkaloids, were combined.

Fraction E.—The mixture of quaternary chlorides was converted into a mixture of quaternary iodide salts with potassium iodide in aqueous solution. The mixture of iodides was fractionated by successive extraction with chloroform, acetone, and methanol. The methanol extract deposited a crude crystalline solid upon concentration. Repeated recrystallization from methanol gave crystalline magnoflorine iodide as pale-yellow prisms, m.p. 251–252°; $[\alpha]_D^{20}$ + 198° (c 0.23, methanol). The melting point was not depressed on admixture with an authentic sample of magnoflorine iodide.[3] The paper chromatographic behavior and infrared spectrum (Nujol mull) were identical with those of the authentic magnoflorine iodide sample.[4]

The Nonquaternary Nonphenolic Fraction.—The crude alkaloid mixture was fractionated by column chromatography on 1 Kg. Florisil. The column was developed by successive elution with benzene, 10, 30, and 60% chloroform-benzene, chloroform, 20% acetone-chloroform, acetone, and methanol. The 30 and 60% chloroform-benzene eluates, on treatment with ethyl acetate, gave 12-Gm. colorless needles, m.p. 160–161°, $[\alpha]_D^{25}$ + 133° (c 0.83, methanol), + 89° (c 0.88, chloroform), $\lambda_{max.}$ 282 mμ, (ε 17,000), 302 mμ (ε 13,000). The N.M.R. spectrum ($CDCl_3$) showed τ = 7.55, 7.52 (6H, N-methyl), 6.40, 6.29, 6.21, 6.19, 6.17, 6.09, 6.05 (21H, O-methyl), 3.79, 3.47, 3.40, 3.37, 3.32, 1.77 (7H aromatic).

Anal.—Calcd. for $C_{41}H_{48}O_8N_2$: C, 70.67; H, 6.94; N, 4.02; 7 (OCH_3), 31.17; 2 (NCH_3), 8.33. Found: C, 70.72; H, 6.72; N, 4.07; (OCH_3), 27.40; (NCH_3), 7.48.

Hofmann Degradation of Thalicarpine

About 3 mg. of anhydrous potassium carbonate was added to a 3-Gm. solution of thalicarpine (4) in 20 ml. methanol and 10 ml. methyl iodide. The solution was heated under reflux on a steam bath for 6 hours. The reaction mixture was then brought to dryness under reduced pressure to yield a semisolid residue. All attempts to crystallize the residue failed. The amorphous thalicarpine dimethiodide (4.29 Gm.) was dissolved in 25 ml. of methanol, and 10 Gm. of potassium hydroxide was added to the solution in small portions to obtain a clear solution. The reaction mixture was heated on a steam bath for 2 hours under magnetic stirring. The mixture was cooled in an ice bath and then evaporated to dryness under reduced pressure. The dried residue was triturated with 50 ml. of water and 50 ml. of ether. The aqueous layer was washed with another portion of 50 ml. ether. The ether layer and wash were combined, washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure to yield 3.36 Gm. of semisolid residue. The Hofmann methine thus obtained was also uncrystallizable. The amorphous Hofmann methine was treated with 10 ml. methyl iodide in 20 ml. methanol at the boiling point of the solution for 6 hours. The reaction mixture was then brought to dryness to leave a 4.05-Gm. brown solid. The residue was treated with methanol to give colorless micro prisms, m.p. 275–276° dec., $[\alpha]_D^{25}$ + 0° (c 1.24, methanol), $\lambda_{max.}$ 266 mμ (ε 62,000), 309 mμ (ε 27,000), 323 mμ (ε 28,000), 346 mμ (ε 20,000).

Anal.—Calcd. for $C_{45}H_{58}O_8N_2I_2 \cdot H_2O$: C, 52.64; H, 5.89; N, 2.73; I, 24.72; 7 (OCH_3), 21.16; 4 (NCH_3), 11.31. Found: C, 52.25; H, 5.59; N, 2.91; I, 25.04; (OCH_3), 21.33; (NCH_3), 11.47.

Thalicarpine dimethylmethine dimethiodide (1.2 Gm.) was converted into methochloride by the action of freshly prepared silver chloride to obtain 1.06 Gm. of the glass-like dimethochloride. A 10-Gm. quantity of potassium hydroxide was added in small portions to a solution of the methochloride in 20 ml. of water and 10 ml. of methanol. The reaction mixture was then heated on a steam bath for 3 hours under

[3] The authors thank Professor E. Fujita, Institute for Chemical Research, Kyoto University, Kyoto, Japan, for an authentic sample of magnoflorine iodide.

[4] Experiment by Dr. B. Dasgupta.

magnetic stirring. The vigorous evolution of trimethylamine gas from the reaction mixture was observed during the early part of the reaction. The reaction mixture was then brought to dryness under reduced pressure. The dried residue was triturated with 50 ml. of water and 200 ml. of ether. The aqueous layer was washed with 100 ml. of ether. The ethereal layer was combined with the washing, washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness to leave a 0.75-Gm. yellow oil. The des-*N*-methine crystallized from ether as colorless fine prisms (0.64 Gm.), m.p. 170–172°, $[\alpha]_D^{25} \pm 0°$ (c 2.05, chloroform); $\lambda_{max.}$ 267 mμ (ε 55,000), 317 mμ (ε 31,000); N.M.R. spectrum ($CDCl_3$), τ = 5.76, 5.83, 5.88, 5.91, 6.05, 6.07, 6.19 (21H, O-methyl).

Anal.—Calcd. for $C_{39}H_{38}O_8$: C, 73.80; H, 6.04. Found: C, 73.54; H, 6.20.

PHARMACOLOGICAL RESULTS[2]

Thalicarpine was evaluated in a variety of pharmacologic procedures and found to possess a modicum of biological activity. In acute dose range or toxicity studies, oral doses of 300 mg./Kg. failed to produce discernible gross behavioral changes in the mouse. Intraperitoneal injection of 25 mg./Kg. of thalicarpine to a cat produced sensitivity of the forepaws, rubbing of the neck, and emesis.

The principal action of thalicarpine on blood pressure was depressor in nature. In the cat anesthetized with chloralose, mean arterial blood pressure was lowered transiently following acute intravenous doses ranging from 0.5 to 5 mg./Kg. Lethality, due to respiratory arrest, occurred at a dose of 10 mg./Kg. Bradycardia, respiratory depression, and andrenergic blocking action accounted for the weak hypotensive activity. Anticoagulant, hypoglycemic, and anticonvulsant properties were not observed after oral doses of 100 mg./Kg. in the rat, guinea pig, or mouse, respectively. An oral dose of 50 mg./Kg. caused a slight antidiuretic response in rats hydrated with saline. In the Randall and Selitto test for anti-inflammatory activity oral doses of 50 mg./Kg. of thalicarpine produced a very low order of analgetic activity (25%) and no significant antipyretic action.

In summary, weak hypotensive activity of a transient nature was the principal action of thalicarpine when injected intravenously into the anesthetized cat. Respiratory toxicity and weak adrenolytic activity accompanied this action. Thalicarpine failed to exhibit significant biological activity as an anti-inflammatory, anticoagulant, hypoglycemic, or diuretic agent.

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ESSENTIAL OILS contain volatile compounds representing many classes of organic substances. One such volatile oil is the well known oil of nutmeg (*Myristica*), an important spice used for the flavoring of numerous food products. It is also used as a component of certain types of perfumes and as a flavoring agent for dentifrices (1). The literature lists two oils of myristica—oil of nutmeg and oil of mace. Both are derived from the fruit of *Myristica fragrans* Houtt. (*fam. Myristicaceae*) (2).

The dried seeds of nutmeg contain from 5 to 15% of the volatile oil, as well as from 25 to 40% of a fixed oil, and from 5 to 15% of ash. The rest consists of moisture, fiber, and starch (3, 4).

There are two principal types of nutmeg which are recognized today, and these depend primarily on geographical origin. "Banda nutmegs" or East Indian variety are the finest; the other variety comes from the West Indies (5).

The West Indian type of oil has a lower specific gravity, lower refractive index, and a lower residue on evaporation, but has a higher

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magnetic stirring. The vigorous evolution of trimethylamine gas from the reaction mixture was observed during the early part of the reaction. The reaction mixture was then brought to dryness under reduced pressure. The dried residue was triturated with 50 ml. of water and 200 ml. of ether. The aqueous layer was washed with 100 ml. of ether. The ethereal layer was combined with the washing, washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness to leave a 0.75-Gm. yellow oil. The des-*N*-methine crystallized from ether as colorless fine prisms (0.64 Gm.), m.p. 170–172°, $[\alpha]_D^{25} \pm 0°$ (c 2.05, chloroform); $\lambda_{max.}$ 267 mμ (ε 55,000), 317 mμ (ε 31,000); N.M.R. spectrum ($CDCl_3$), τ = 5.76, 5.83, 5.88, 5.91, 6.05, 6.07, 6.19 (21H, O-methyl).

Anal.—Calcd. for $C_{39}H_{38}O_8$: C, 73.80; H, 6.04. Found: C, 73.54; H, 6.20.

PHARMACOLOGICAL RESULTS[2]

Thalicarpine was evaluated in a variety of pharmacologic procedures and found to possess a modicum of biological activity. In acute dose range or toxicity studies, oral doses of 300 mg./Kg. failed to produce discernible gross behavioral changes in the mouse. Intraperitoneal injection of 25 mg./Kg. of thalicarpine to a cat produced sensitivity of the forepaws, rubbing of the neck, and emesis.

The principal action of thalicarpine on blood pressure was depressor in nature. In the cat anesthetized with chloralose, mean arterial blood pressure was lowered transiently following acute intravenous doses ranging from 0.5 to 5 mg./Kg. Lethality, due to respiratory arrest, occurred at a dose of 10 mg./Kg. Bradycardia, respiratory depression, and andrenergic blocking action accounted for the weak hypotensive activity. Anticoagulant, hypoglycemic, and anticonvulsant properties were not observed after oral doses of 100 mg./Kg. in the rat, guinea pig, or mouse, respectively. An oral dose of 50 mg./Kg. caused a slight antidiuretic response in rats hydrated with saline. In the Randall and Selitto test for anti-inflammatory activity oral doses of 50 mg./Kg. of thalicarpine produced a very low order of analgetic activity (25%) and no significant antipyretic action.

In summary, weak hypotensive activity of a transient nature was the principal action of thalicarpine when injected intravenously into the anesthetized cat. Respiratory toxicity and weak adrenolytic activity accompanied this action. Thalicarpine failed to exhibit significant biological activity as an anti-inflammatory, anticoagulant, hypoglycemic, or diuretic agent.

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optical rotation than the oil obtained from the East Indies.

Nutmeg oil consists principally of terpenes, and about 60% of the oil distils below 180° (6). In 1907, Power and Salway (7) examined a "genuine" oil of Ceylon nutmeg having a specific gravity of 0.8690 at 15° and an optical rotation of 38°4′. They found this oil to contain primarily over 80% of terpenes and 6% terpene alcohols.

In reviewing the literature concerning differences between East Indian and West Indian oil, one finds statements primarily referring to the higher content of terpenes and a lower content of phenols in the West Indian oil. Therefore, it was of interest to undertake an analysis of the nutmeg oil obtained from these two sources, using gas chromatography.

EXPERIMENTAL

Apparatus.—The apparatus employed was Beckman GC-2 gas chromatograph with a four-filament thermal conductivity cell connected to Sargent SR recorder equipped with a K-4 disk integrator. Helium was used as the carrier gas, and the flow rate was maintained at 38 ml. per minute. The filament current used was 380 ma., while the chart speed of the recorder was 1.0 in. per minute.

Column Preparation.—Reoplex 400 (polyethylene glycol adipate) was used as the stationary liquid phase and was supported on 80/100-mesh Diachromite. Approximately 20.0 Gm. of the solid support was required to pack a 6-ft. column. The 5-Gm. liquid phase was introduced by deposition gradually and with constant stirring from a 45 ml. solution in dichloromethane. The resulting mixture was first dried in air, then at 90° in the oven for about 3 hours. Columns used were of copper tubing, 1/4 in. diam. and 6 ft. in length.

Operating Conditions.—Temperatures ranging from 100 to 190° were employed. Samples of 0.005 ml. were used, except in cases where the fraction was collected. Then the sample was increased to 0.05 ml.

Oil Samples and Standard Compounds.—The four commercially available samples of nutmeg oil that were analyzed are listed below along with their respective specific gravities and refractive indices.[1] Sample A (East Indian nutmeg oil U.S.P. extra) n_D^{20}, 1.4804; sp. gr.^{24}, 0.884. Sample B (East Indian nutmeg oil U.S.P.) n_D^{20}, 1.4809; sp. gr.^{24}, 0.897. Sample C (East Indian nutmeg oil U.S.P.) n_D^{20}, 1.4793; sp. gr.^{24}, 0.880. Sample D (West Indian nutmeg oil U.S.P.) n_D^{20}, 1.4756; sp. gr.^{24}, 0.870.

For purposes of identification the retention times and relative retention times of the unknown were compared with a known pure standard sample. In this way a tentative identification of most of the compounds could be made. We have confirmed these particular results by enrichment method, in which known compounds were added individually to the sample chromatographed and compared in each instance to the chromatogram obtained by using the oil alone. Infrared spectra were obtained for unknown peaks. Tables I, II, and III list the retention times and relative retention times of standard compounds at 100 and 160°, respectively. Relative retention times are based on *p*-cymene.

[1] The authors express their thanks to Fritzsche Brothers, Inc., New York, N. Y., for supplying Sample C of oil of nutmeg.

TABLE I.—RETENTION TIMES AND RELATIVE RETENTION TIMES OF STANDARD COMPOUNDS ON REOPLEX 400 AT 100°C. (FLOW RATE, 38 ML./MIN.; FILAMENT CURRENT, 380 MA.; *p*-CYMENE = 1.00)

Standard Compd.	Retention Times, min.	Relative Retention Times
dl-α-Pinene	3.36	0.25
d-Camphene	4.56	0.34
β-Pinene	5.56	0.41
Terpinolene	7.56	0.56
d-Limonene	8.80	0.65
p-Cymene	13.5	1.00
dl-Linalool	52.4	3.88
Camphor	54.0	3.99
dl-α-Terpineol	129	9.51

TABLE II.—RETENTION TIMES AND RELATIVE RETENTION TIMES OF STANDARD COMPOUNDS ON REOPLEX 400 AT 130°C. (FLOW RATE, 38 ML./MIN.; FILAMENT CURRENT, 380 MA.; *p*-CYMENE = 1.00)

Standard Compd.	Retention Times, min.	Relative Retention Times
dl-α-Pinene	1.89	0.33
d-Camphene	1.95	0.34
β-Pinene	3.02	0.51
Terpinolene	3.69	0.65
d-Limonene	4.24	0.72
p-Cymene	5.70	1.00
dl-Linalool	16.6	2.90
Camphor	19.2	3.37
dl-Borneol	28.9	5.06
dl-α-Terpineol	34.7	6.09
Geraniol	67.3	11.8
Safrole	80.7	14.2
Eugenol (was not eluted even after 125 min.)		

TABLE III.—RETENTION TIMES AND RELATIVE RETENTION TIMES OF STANDARD COMPOUNDS ON REOPLEX 400 AT 160°C. (FLOW RATE, 38 ML./MIN.; FILAMENT CURRENT = 380 MA.; *p*-CYMENE = 1.00)

Standard Compd.	Retention Times, min.	Relative Retention Times
dl-α-Pinene	1.32	0.42
d-Camphene	1.32	0.42
β-Pinene	1.88	0.59
Terpinolene	2.22	0.70
Limonene	2.44	0.78
p-Cymene	3.18	1.00
dl-Linalool	7.00	2.20
Camphor	9.53	3.00
dl-α-Terpineol	14.6	4.58
Geraniol	24.9	7.55
Safrole	30.5	9.62
Eugenol	83.0	26.1
Isoeugenol	146	46.1

RESULTS AND DISCUSSION

Three commercial samples of an East Indian nutmeg oil and one sample of a West Indian nutmeg oil were analyzed by gas liquid partition chromatography. Of a number of stationary liquid phases that were used, only Reoplex 400 supported on 80/100-mesh Dichromite gave satisfactory results.

That the various components of the nutmeg oils could be separated more efficiently at different temperatures is shown in Figs. 1–3 when Sample A of oil of nutmeg was chromatographed at 100, 130, and 160°, respectively. For example, eight peaks were observed at 100° (Fig. 1), while at 130° the number of peaks increased to ten (Fig. 2). At 160°, two additional peaks were obtained using this same oil (Fig. 3). Similar results were observed with the other samples of the East Indian variety oil.

Attention should be directed to Fig. 2 which shows ten peaks obtained with Sample A at 130°. Comparing this with the chromatograms representing the peaks obtained at 100° (Fig. 1) and 160° (Fig. 3), respectively—and using this same oil—it should be pointed out that in the chromatogram obtained at 100°, peak numbers 8, 9, 10, 11, and 12 (Fig. 3), representing higher retention times, are absent. It was further observed that peak number 3 at 130° (Fig. 2) or 160° (Fig. 3) could be resolved into two components at 100°. One of these peaks (No. 3) we have identified as limonene.

We have used the results obtained at 100 and 160°, respectively, to calculate the percentages of the components of the East Indian oils (Samples A, B, and C). With the West Indian oil (Sample D) two unidentified components which appeared as two distinct small peaks at 130° (Fig. 5) but only as one peak at 160° (Fig. 6) were observed. At temperature of 100° (Fig. 4) these same constituents also appeared as two peaks, but one of them (with the lower retention time) could not be separated distinctly from the preceding one. The results obtained at 130° were also used to calculate the percentages of these two components of the oil. We have based all the percentages as calculated on the total eluted. To keep the peaks within the limits, it is customary to change attenuation at times from component to component. This was done in this investigation also. It is well recognized, however, that a particular area will differ with change in attenuation. Since the percentages are calculated from the integrated signals, and since these signals are proportional to the attenuation, each sample of the oil was re-run at one and the same attenuation.

The samples of the East Indian oil of nutmeg (Samples A, B, and C) that were examined differed in composition not only from the West Indian oil, but they also showed variation in composition from one to the other (Table IV).

All of the oils of the East as well as the West Indian variety were found to contain α-pinene, β-pinene, and limonene. Of the four samples of oil that we have investigated, the West Indian variety contained the highest total percentage (40.1%) of these terpenes, while the East Indian Samples A, B, and C contained 30.9, 30.2, and 33.9-

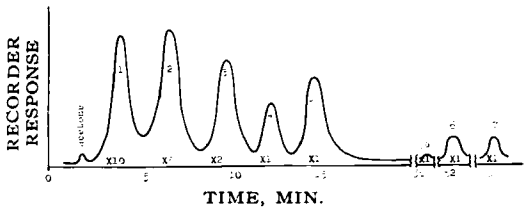

Fig. 1.—Typical chromatogram of Sample A of nutmeg oil on Reoplex 400 at 100°C.

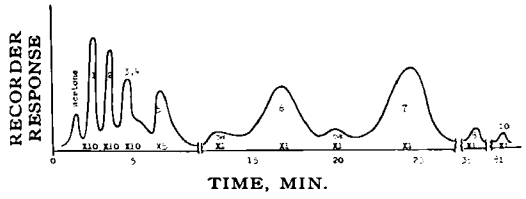

Fig. 2.—Typical chromatogram of Sample A of nutmeg oil on Reoplex 400 at 130°C.

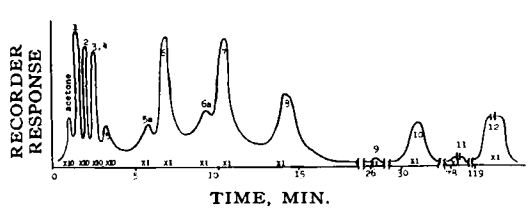

Fig. 3.—Typical chromatogram of Sample A of nutmeg oil on Reoplex 400 at 160°C.

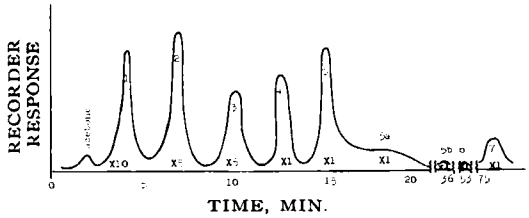

Fig. 4.—Typical chromatogram of Sample D of nutmeg oil on Reoplex 400 at 100°C.

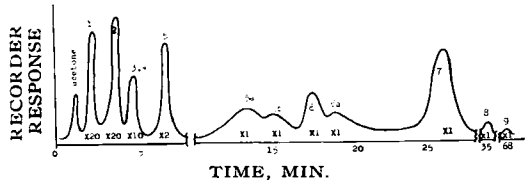

Fig. 5.—Typical chromatogram of Sample D of nutmeg oil on Reoplex 400 at 130°C.

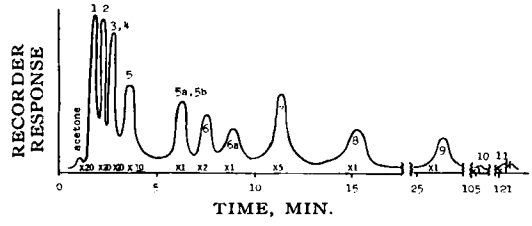

Fig. 6.—Typical chromatogram of Sample D of nutmeg oil on Reoplex 400 at 160°C.

TABLE IV.—COMPOSITION OF NUTMEG OILS, PER CENT

	Sample A	Sample B	Sample C	Sample D
α-Pinene	13.9	12.9	12.5	13.4
β-Pinene	12.3	12.6	19.1	14.8
Terpinolene	...	1.4	0.8	...
Limonene	4.7	4.7	2.3	11.9
p-Cymene	1.6	2.6	2.7	4.3
Linalool	10.6	8.1	...	5.9
Camphor	...	...	...	3.4
Terpinen-4-ol	14.5	14.2	19.5	24.7
α-Terpineol	7.7	4.7	4.0	5.0
Geraniol	0.3	11.9	...	3.6
Safrole	6.0	4.2	5.5	...
Eugenol	1.3	3.8	0.3	...
Unknown A	22.2	13.9	25.9	1.7
Unid. (3 components)	4.9	5.4	...	...
Unid. (4 components)	...	...	7.4	11.4

%, respectively. Table IV lists the percentages of individual components in each of the oils. The presence of terpinolene was observed only in Samples B and C and in 1.4 and 0.8%, respectively. One of the peaks which appeared on each of the chromatograms was identified as p-cymene and ranged from 1.6 to 4.3% (Table IV).

It should be noted that linalool and geraniol are absent in Sample C of the East Indian nutmeg oil. Samples A, B, and D contain linalool in 10.6, 8.1, and 5.9%, respectively. Geraniol was found to be present in two samples of the East Indian oil, Samples A and B. It is interesting that there is a large difference in percentage of geraniol in the two oils—namely, 0.3% in Sample A and 11.9% in Sample B. In the West Indian oil, 3.6% geraniol was found.

A component with relative retention times ranging from 5.48 to 5.58 at 100°, 4.15 to 4.31 at 130°, and 3.22 to 3.26 at 160° (Tables V, VI, VII, and VIII) which was present in all the oils, was collected and an infrared spectrum was obtained. By comparison of I.R. spectra, this compound is tentatively identified as terpinen-4 ol. West Indian nutmeg oil contained the highest amount of terpinen-4 ol (24.7%), while East Indian oils as analyzed contained the following percentages of this alcohol: 14.5, 14.2, and 19.5% for Samples A, B, and C, respectively. As can be seen from Figs. 2 and 5, another component is present with a higher retention time (peak 8). This is present in approximate range of 4–8%, depending upon the oil; it has been tentatively identified as α-terpineol using the enrichment procedure.

While safrole and eugenol were absent in the West Indian oil, both of these were present in all three samples of the East Indian variety. The per cent of safrole ranges from 4.2 to 6.0 and that of eugenol from 0.3 to as high as 3.8% (Table IV).

Another component (Unknown A) found in all samples of oil tested had a relative retention time ranging from 37.3 to 38.6 (Tables V, VI, VII, and VIII) at 160°. It was collected and its infrared

TABLE V.—RELATIVE RETENTION TIMES OF SAMPLE A OF EAST INDIAN NUTMEG OIL. (STATIONARY PHASE-REOPLEX 400; HELIUM FLOW RATE 38 ML./MIN. p-CYMENE = 1.00; FIGS. 1, 2, AND 3)

Peak No., Compd.	100°C. Unknown	100°C. Known	130°C. Unknown	130°C. Known	160°C. Unknown	160°C. Known
1. α-Pinene	0.25	0.25	0.32	0.33	0.42	0.42
2. β-Pinene	0.42	0.41	0.50	0.51	0.60	0.59
3. Limonene	0.66	0.65	0.71	0.72	0.78	0.78
4. Unidentified	0.86	...	...	...	...	...
5. p-Cymene	1.00	1.00	1.02	1.00	0.99	1.00
5a. Unidentified	2.67	...	2.04	...	1.82	...
6. Linalool	3.88	3.88	2.86	2.90	2.16	2.20
6a. Unidentified	...	...	3.49	...	2.74	...
7. Terpinen-4-ol	5.55	...	4.15	...	3.22	...
8. α-Terpineol	...	...	6.10	6.09	4.45	4.58
9. Geraniol	...	...	...	...	8.00	7.55
10. Safrole	...	...	14.2	14.2	9.24	9.62
11. Eugenol	...	...	...	...	22.5	26.1
12. Unknown A	...	...	...	...	37.3	...

TABLE VI.—RELATIVE RETENTION TIMES OF SAMPLE B OF EAST INDIAN NUTMEG OIL. (STATIONARY PHASE-REOPLEX 400; HELIUM FLOW RATE 38 ML./MIN. p-CYMENE = 1.00)

Peak No., Compd.	100°C. Unknown	100°C. Known	130°C. Unknown	130°C. Known	160°C. Unknown	160°C. Known
1. α-Pinene	0.24	0.25	0.33	0.34	0.41	0.42
2. β-Pinene	0.42	0.41	0.53	0.51	0.59	0.59
2a. Terpinolene	0.60	0.56	...	...	...	...
3. Limonene	0.64	0.65	0.75	0.72	0.79	0.78
4. Unidentified	0.84	...	...	...	...	...
5. p-Cymene	1.00	1.00	1.06	1.00	1.05	1.00
5a. Unidentified	2.68	...	2.08	...	1.86	...
6. Linalool	3.79	3.88	2.88	2.90	2.20	2.20
6a. Unidentified	...	...	3.55	...	2.80	...
7. Terpinen-4-ol	5.48	...	4.22	...	3.23	...
8. α-Terpineol	...	...	6.18	6.09	4.41	4.58
9. Geraniol	...	...	11.9	11.8	7.79	7.55
10. Safrole	...	...	14.3	14.2	9.53	9.62
11. Eugenol	...	...	...	...	24.2	26.1
12. Unknown A	...	...	...	...	38.6	...

spectrum was obtained. The spectrum showed absorption bands usually associated with aromatic ring, 1630 cm.$^{-1}$, 1516 cm.$^{-1}$, 1459 cm.$^{-1}$, and a carbonyl group 1718 cm.$^{-1}$ The quantity of this component ranged from a low of 1.7% in the West Indian oil to a high of 25.9% in Samples C of East Indian variety (Table IV).

Results obtained by the authors differ from those reported for the oil of nutmeg not only qualitatively but also quantitatively, with the possible exception of trace amounts of the esters of fatty acids, which we did not observe under the conditions used. Myristic acid and myristicin which have been reported present in a concentration of 2–3% were not eluted even after 105 minutes at a higher temperature (190°) than that used for the final analysis of the other components of the oils.

In most instances Power and Salway (8) listed the combined percentages of a few components. For example, according to these investigators α-pinene and *d*-camphene make up about 80% of the oil. Others have reported the presence of about 70% of terpenes (α-pinene, *d*-camphene, β-pinene, and dipentene). Analysis of the four samples of oil examined by us confirmed the presence of α-pinene. Samples A, B, C, and D were observed to contain 13.9, 12.9, 12.5, and 13.4% of α-pinene, respectively, while *d*-camphene was not present.

Only a small amount of β-pinene was detected in an oil analyzed in another study (9). The percentages of β-pinene observed in the present study differ considerably. As can be seen from Table IV, the percentage of β-pinene varies from a low of 12.3% to a high of 19.1%.

About 8% of a substance called dipentene was reported present in the oil analyzed by Power and Salway. It is important to note that this substance identified as dipentene by them (8) is not a pure compound but rather a mixture composed of $C_{10}H_{16}$ terpenes. Westaway and Williams (10) have analyzed two synthetic and two natural samples labeled as dipentene. Both types of samples differed both qualitatively and quantitatively. One natural sample contained as high as 80% of limonene; in the other three samples, limonene was present in various lower concentrations. Terpinolene was observed in varying percentages in all four samples. Other terpenes which were reported present by Westaway and Williams in some samples and absent in others include α-pinene, *d*-camphene, β-pinene, tricyclene, α- and γ-terpinene.

A peak labeled number three (Fig. 1) in this study present in each of the four oils and identified as limonene is the main component of dipentene referred to by Power and Salway. Furthermore, Samples B and C contained small amounts of terpinolene which were not reported present in the oil of nutmeg.

Power and Salway (9) identified *d*-linalool, *d*-borneol, "isoterpineol," and geraniol in nutmeg oil. They reported these four alcohols present in about a total of 6%. In this investigation borneol was absent in all samples of nutmeg oil, while another

TABLE VII.—RELATIVE RETENTION TIMES OF SAMPLE C OF EAST INDIAN NUTMEG OIL. STATIONARY PHASE-REOPLEX 400; HELIUM FLOW RATE 38 ML./MIN. *p*-CYMENE = 1.00

Peak No., Compd.	100°C.		130°C.		160°C.	
	Unknown	Known	Unknown	Known	Unknown	Known
1. α-Pinene	0.23	0.25	0.35	0.34	0.42	0.42
2. β-Pinene	0.41	0.41	0.54	0.51	0.59	0.59
2*a*. Terpinolene	0.63	0.56	...	...	...	...
3. Limonene	0.70	0.65	0.74	0.72	0.80	0.78
4. Unidentified	0.86	...	...	...	...	...
5. *p*-Cymene	1.02	1.00	1.03	1.00	1.07	1.00
6. Unidentified	...	...	2.12	...	1.30	...
6*a*. Unidentified	...	...	2.61	...	2.14	...
6*b*. Unidentified	...	...	3.04	...	2.57	...
7. Terpinen-4-ol	5.58	...	4.31	...	3.26	...
8. α-Terpineol	...	...	6.45	6.09	4.56	4.58
9. Safrole	...	...	14.7	14.2	9.53	9.68
10. Eugenol	...	...	...	...	24.3	26.1
11. Unknown A	...	...	...	...	38.6	...

TABLE VIII.—RELATIVE RETENTION TIMES OF SAMPLE D OF WEST INDIAN NUTMEG OIL. (STATIONARY PHASE-REOPLEX 400; HELIUM FLOW RATE 38 ML./MIN. *p*-CYMENE = 1.00; FIGS. 4, 5, AND 6)

Peak No., Compd.	100°C.		130°C.		160°C.	
	Unknown	Known	Unknown	Known	Unknown	Known
1. α-Pinene	0.24	0.25	0.32	0.33	0.42	0.42
2. β-Pinene	0.43	0.41	0.53	0.51	0.59	0.59
3. Limonene	0.66	0.65	0.72	0.72	0.78	0.78
4. Unidentified	0.85	...	...	...	...	...
5. *p*-Cymene	1.01	1.00	1.01	1.00	1.05	1.00
5*a*. Unidentified	1.30	...	2.13	...	1.86	...
5*b*. Unidentified	2.69	...	2.53	...	...	...
6. Linalool	3.91	3.88	2.92	2.90	2.20	2.20
6*a*. Camphor	...	...	3.31	3.37	2.67	3.00
7. Terpinen-4-ol	5.53	...	4.26	...	3.25	...
8. α-Terpineol	...	...	6.12	6.09	4.61	4.58
9. Geraniol	...	...	11.9	11.8	7.78	7.55
10. Unidentified	...	...	...	...	33.2	...
11. Unknown A	...	...	...	...	38.2	...

component, camphor, was detected in the West Indian oil. Linalool was present in Samples A (10.6%), B (8.1%), and D (5.9%), while it was not observed in Sample C of East Indian variety. The same observation was made as far as geraniol is concerned. We found 3.6% present in the West Indian sample, while two samples of the East Indian variety contain 0.3 and 11.9%, respectively.

The amount of α-terpineol varies depending on the sample of the oil (Table IV). Westway and Williams (10) did not report the percentage of α-terpineol in the oil they investigated.

In 1907 (9), safrole was reported present in about 0.6% in the oil of Ceylon nutmeg. Our analysis confirmed its presence in the East Indian oils only. The amount ranges from 4.2 to 6.0%. This same report mentions the presence of about a total of 0.2% of eugenol and isoeugenol in the oil from Ceylon. A peak corresponding to the retention time of isoeugenol was not observed in the chromatograms. We have observed eugenol in varying amounts in all three samples of the East Indian variety. Samples A, B, and C contain 1.3, 3.8, and 0.3%, respectively, of eugenol.

One of the constituents of the oil, which was not yet reported in literature, was isolated from the oil, using gas chromatography; an infrared spectrum was run. This constituent, which ranges from as low as 1.7% in the West Indian sample to as high as 25.9% in Sample C of the East Indian variety, was tentatively labeled as "Unknown A."

SUMMARY

Four commercially available samples of nutmeg oil were analyzed by gas-liquid chromatography, using 20% Reoplex 400 column. Relative retention times and enrichment procedure were used to identify the constituents of the oils. In some instances infrared spectra were employed.

The following components were present in the three samples of East Indian variety and in the West Indian sample: α-pinene, β-pinene, limonene, *p*-cymene, terpinen-4 ol, α-terpineol, and Unknown A. Terpinolene was found in small amounts and only in two samples of East Indian oil, while linalool and geraniol are absent in one sample of East Indian nutmeg oil. Safrole and eugenol were present in all three samples of the East Indian variety but were not observed in the West Indian sample. Camphor was found in the West Indian oil only.

Differences are also observed between the percentages of the components present not only compared to those listed in the literature but also between the oils analyzed.

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ERRATUM

In the paper titled "Absorption, Metabolism, and Excretion of the Semisynthetic Penicillin 6 (2-Ethoxy-1-naphthamido)penicillanic Acid (Nafcillin)" (1), a broken line represents intramuscular and a solid line represents oral groups of dogs in Figs. 1–3.

(1) Walkenstein, S. S., Wiser, R., LeBoutillier, E., Gudmundsen, C., and Kimmel, H., THIS JOURNAL, **52**, 763 (1963).

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Pharmaceutical Applications of the Concept of Equilibrium Moisture Contents

By MORTON W. SCOTT, HERBERT A. LIEBERMAN, and FRANK S. CHOW

Equilibrium moisture content (EMC) data have been obtained for two representative tablet granulations. These data are useful in analysis of drying operations and particularly in predicting final moisture contents obtainable in "dried" products. The effects of drying variables, such as relative humidity and inlet air temperature, on product moisture levels are established. Similarly, the influence of storage conditions on moisture gain is predictable. Data obtained from drying studies with a lactose placebo granulation are used to illustrate the relationship between equilibrium moisture content values and minimum required drying times. The importance and economic advantages obtainable by review of this type data before establishment of moisture specifications for dried materials is illustrated. In addition, equilibrium moisture content values for several tablet fillers, disintegrants, and other pharmaceuticals are presented. Based on these preliminary studies, the EMC concept may have useful applications in the design of moisture stable products.

INTRODUCTION

ADJUSTMENT AND CONTROL of moisture levels is of general importance in the development and manufacture of superior pharmaceutical products. The difficulties encountered in establishing proper levels of moisture can be broadly divided into two categories. The first category includes problems connected with processing and handling operations, such as drying and bulk powder filling, etc. Those problems which relate more directly to formulation design, such as the stabilization of moisture sensitive materials, are included in the second category. Unfortunately, proper attention has not been devoted to either of these problem areas in the pharmaceutical literature.

Scattered reports are available which deal with the effects of moisture on specific pharmaceuticals. Leeson and Mattocks (1) have examined the stability of aspirin stored under elevated humidity conditions. These workers present kinetic interpretations of the data and show that decomposition is dependent on the aqueous vapor pressure and storage temperature. The stability of ascorbic acid at high relative humidities has been studied by de la Vega (2), while Blaug, *et al.* (3), discussed methods of preparing ascorbic acid tablets with reduced moisture sensitivity. The adverse influence of moisture on the flowability of pharmaceutical powders also has been demonstrated (4). The report of Craik and Miller on this subject is noteworthy (5). Attention has been paid also to the moisture vapor transmission characteristics of packaging components used for pharmaceuticals (6). A generalized approach to the problems of moisture control—capable of leading to the improvement of formulation techniques, processing operations, and storage procedures—has not been developed yet.

The present study was undertaken to explore the potential usefulness of the equilibrium moisture content concept as applied to materials of pharmaceutical interest. This concept serves as a basic tool in the design of drying, air conditioning, and humidification operations (7) and would seem to represent a correct starting point for the study of moisture relationships in pharmaceutical products.

EQUILIBRIUM MOISTURE CONTENT

If a solid is exposed to a continual supply of air at constant temperature and relative humidity, the solid will either lose moisture by evaporation or gain moisture until an equilibrium condition is obtained. The moisture present in the solid at this point is defined as the equilibrium moisture content (EMC) under the given exposure conditions. Rigorous thermodynamic treatments of the equilibrium state have appeared in the literature (8, 9). For all cases of practical importance, equilibrium will be established when the vapor pressure exerted by the moisture in the solid equals the partial pressure of the water vapor in the air. A new equilibrium moisture content results with a change in the partial pressure of water in the air. It is customary to

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express these partial pressures in terms of relative humidities (R.H.) and to express the moisture contents of the solid on a dry basis. The collection of such EMC–relative humidity data can be used to prepare an entire moisture equilibrium curve for the solid. The EMC relationship for a hypothetical hygroscopic solid is shown in Fig. 1.

Point X_S in Fig. 1 represents the EMC of the solid after exposure to air at 100% R.H. Since the partial pressure of moisture in air at this humidity is equal to the vapor pressure of liquid water, all moisture contents above X_S consist in part of liquid water capable of exerting its full vapor pressure. The moisture level between X_S and some higher level X_H is the "unbound moisture" of the solid.

At all moisture contents less than X_S, the moisture present is not capable of exerting its full vapor pressure. This water is "bound" within or on the solid and may be held by chemical or physical adsorption, in capillaries, as the solvent in soluble portions of the solid, or by other mechanisms (7).

Point X_E represents the EMC of the solid after exposure to air at 50% R.H. X_E is the limiting level of "dryness" which can be achieved by treating the solid (initially at some higher moisture content, such as X_H) with air at 50% R.H. The difference between X_H and X_E is defined as the "free water" content of the solid (7).

Equilibrium moisture content curves have been reported for numerous materials including dehydrated foods (10), cereal grains (11, 12), soils (13), and cotton, wood, and leather (14). These EMC curves vary greatly with the type of solid examined. For example, nonporous insoluble solids such as zinc oxide generally show relatively low EMC values over a wide range of relative humidities (14). Fibrous, cellular, and porous solids exhibit broader variations in their EMC levels with changes in relative humidity. Hysteresis effects have been reported (15), and equations describing the adsorption isotherm have been applied (8, 10). Methods for conducting the EMC determinations by static and dynamic (forced convection) techniques have been adequately reviewed in the literature (8).

EXPERIMENTAL

Materials.—Equilibrium moisture content curves were established for the following categories of materials. U.S.P. or N.F. grades were used where specific sources of supply are not listed.

Tablet Fillers: Mannitol, lactose, terra alba (extra pure grade, C. Chrystal and Co.), kaolin, sucrose, and dibasic calcium phosphate.

Tablet Disintegrators: Starch, guar gum (grade A-20-D, Steinhall and Co.), Solka Floc (Brown Co.), and bentonite (C. Chrystal and Co.).

Binding Agents: Ethylcellulose, 10 cps.; methylcellulose, 15 and 400 cps.

Tablet Lubricants: Talc, stearic acid, and magnesium stearate.

Tablet Granulations and Tablets:

Placebo granulation (12–20 mesh) consisting of 85% lactose, 5% methylcellulose (400 cps.) and 10% starch (wet granulated with water).

Antacid granulation (12–20 mesh) based on magnesium trisilicate and wet granulated with syrup.

Ferrous sulfate tablets consisting of 8% ferrous sulfate, 5% anhydrous calcium chloride, 86% of gelatin–terra alba filler, and 1% magnesium stearate compressed to a hardness of 6, 8, and 10 Strong-Cobb units.

Fifty-milligram ascorbic acid tablets containing 86% of gelatin–terra alba filler and 1% magnesium stearate.

Procedure.—Static EMC determinations were made by placing samples of each material into desiccators containing one of the following saturated solutions: ammonium dihydrogen phosphate (93% R.H.), ammonium sulfate (81.1% R.H.), ammonium chloride plus potassium nitrate (71.2% R.H.), magnesium nitrate hexahydrate (52% R.H.), potassium carbonate dihydrate (43% R.H.), and calcium chloride hexahydrate (31.0% R.H.). These specific agents were chosen because the relative humidity established above the saturated solution is relatively constant over the (narrow) range of temperature (25 ± 1°) encountered in this study.

Approximately 15 Gm., or 50–100 tablets, of each test material was used as the sample. Weight gains were recorded after various intervals of storage. When constant weight was achieved, L.O.D. was determined using the Cenco moisture balance (90 volts for 7 minutes). These L.O.D. values were converted to moisture contents (dry basis) and plotted *versus* relative humidity to yield the equilibrium moisture content curves.

Assays for ferrous sulfate and ascorbic acid were done according to the U.S.P. methods.

RESULTS AND DISCUSSION

Pharmaceutical Engineering Considerations.—Figure 2 illustrates the EMC curves for the antacid and placebo tablet granulations. It is recognized that the antacid granulation is far more sensitive to changes in relative humidity than the placebo formulation. The variations in EMC curves obtainable with different materials is clearly illustrated by these data.

EMC relationships (as illustrated in Fig. 2) have immediate usefulness in the selection of proper drying conditions for tablet granulations. For example, the EMC for the placebo granulation at 40% R.H. is approximately 1%. Once this equilibrium moisture content has been reached, no further reduction in moisture content can be achieved by further drying with air at 40% relative humidity. Thus, the moisture contents of tablet granulations during the course of actual drying cycles can be compared to the EMC value to determine when the granulation has reached "dryness." This information can then be used to establish the minimum drying time required under the particular process conditions employed.[1]

The effects of changes in the relative humidity of the drying air on the moisture content of the granulation also are obtainable from Fig. 2. With the antacid granulation, a significant decrease in moisture content is observed with decreasing R.H. With the placebo, however, a decrease from 70 to 30% R.H. has little or no effect on final moisture

[1] For dryer calculations, it is preferable to determine EMC values from moisture desorption studies. The use of adsorption data may introduce slight errors if hysteresis effects are present.

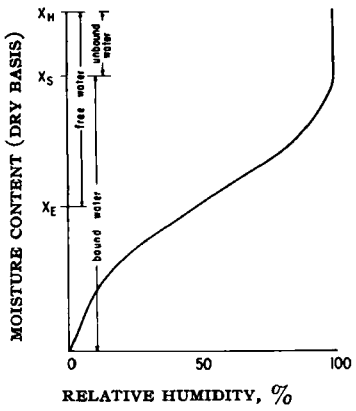

Fig. 1.—EMC curve for a hypothetical hygroscopic solid.

content. If the level of moisture finally present in the placebo granulation after drying with air at 70% R.H. adversely influenced compression characteristics, little improvement would be obtained by drying with air at 30% R.H. This illustrative example can be extended to other properties of the granulation (which are moisture content dependent) such as flow behavior, friability, caking tendencies, etc.

In the case of tray dryers and other units which have heating elements external to the drying chamber, estimates of the EMC at various elevated temperatures can be obtained. This is accomplished by first determining, from psychrometric charts (7), the decrease in relative humidity produced by heating the air to the desired temperature. The equilibrium moisture content of the solid at the elevated temperature is then read from the EMC curve using the new R.H. value as the parameter.[2] These calculations are concerned only with factors of equilibrium moisture contents and do not relate to any changes in the rates of drying which result with changes in R.H. or air temperature. Detailed analyses of these latter factors have appeared in the literature (7).

The EMC curves also have utility in establishing the influence of storage conditions on the moisture content of the product. It is clear that products dried to one moisture level under a given set of drying (or storage) conditions will assume new moisture content values if stored under conditions of different R.H. In the case of the antacid granulation illustrated in Fig. 2, about 9% moisture will be present after storage at 70% R.H. regardless of the moisture content obtained in previous drying operations.[3] No attempt should be made in the drying operations, therefore, to reduce the moisture content of the granulation lower than 9% if the product is subsequently stored at 70% R.H. for protracted intervals.

The compression and flow characteristics of many tablet granulations are influenced by relatively small variations in moisture content. Since quantities of moisture may be adsorbed during "in process" storage, the moisture specifications governing acceptability of such granulations should be based at a level at which successful tableting occurs and not on the moisture content obtained immediately after drying. Limits on the moisture content of the granulations leaving the dryer can be applied, of course, for control of dryer performance.

The moisture level specified for the dry granulation has an important influence on the "throughput" and efficiency of a given dryer. For products

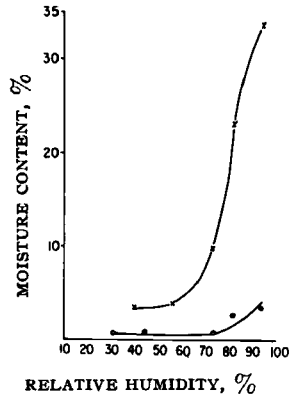

Fig. 2.—EMC curves for placebo and antacid tablet granulation. Key: ×—× antacid, O—O placebo.

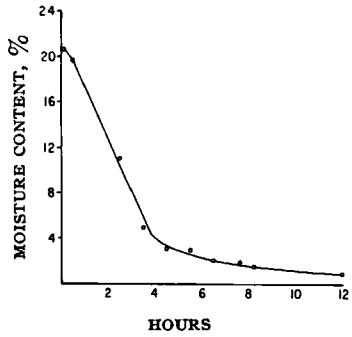

Fig. 3.—Moisture content data for tray drying placebo granulation (inlet air at 104°).

dried to their EMC, drying rates may decrease significantly as the EMC is approached. Thus, disproportionately large amounts of time are required to remove the last amounts of free water. This is illustrated in Fig. 3 with data obtained in tray-drying studies with the placebo. In this case, approximately 6 hours of the total drying time of 10–12 hours was utilized in reducing the moisture content from 3 to 1%— a reduction of only 2%. If it were determined that the granulation was actually acceptable at the 3% moisture content, the drying cycle could be reduced by 6 hours. This would double the output of the tray dryer. Overall thermal efficiency would be increased by an even higher factor. Further, during the last 6 hours of drying, the product temperature will be near that of the inlet air so that elimination of this interval may minimize product decomposition during the drying cycle.

Formulation Considerations.—Figure 4 presents

[2] Temperature has a slight humidity-independent effect on EMC (8). This can be neglected in first approximations with most systems.

[3] This assumes that the storage at 70% R.H. is of sufficient duration for equilibrium to be established. For shorter storage times the moisture content of the product may adjust only partially. Hysteresis effects also are assumed to be negligible.

the EMC curves for representative tablet disintegrators. The high EMC sensitivity of these materials to changes in relative humidity is apparent. These EMC characteristics are not surprising since tablet disintegrators, as a class, comprise agents which are chosen (in a sense) for their water adsorptive abilities. No attempt to select the "best" disintegrators on the basis of EMC criteria was made since other factors also influence their performance.

EMC curves for three modified cellulose tablet binders are shown in Fig. 5. The water insoluble

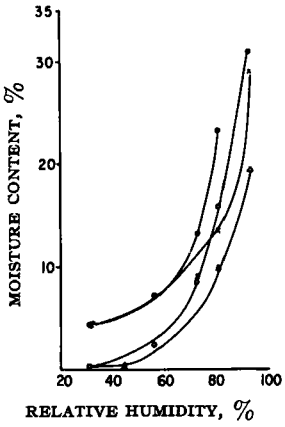

Fig. 4.—EMC curves for tablet disintegrators. Key: Δ—Δ starch, ×—× Solka Floc, O—O guar gum (A-20-D), ●—● bentonite.

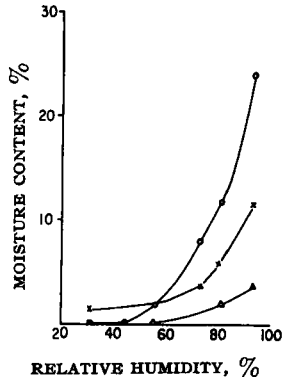

Fig. 5.—EMC curves for tablet binders. Key: O —O methylcellulose (400 cps), × —× methylcellulose (15 cps), Δ —Δ ethylcellulose, (15 cps).

ethylcellulose shows low moisture levels which are sensitive nevertheless to changes in relative humidity. Both grades of methylcellulose have high EMC values, and clear differentiation between the two grades is observed.

Lubricants examined in this study included talc, magnesium stearate, and stearic acid. EMC values for the first two materials were less than 0.5% at all test humidities. Slightly higher results of about 1% were observed for stearic acid. It is probable, however, that this moisture value includes a significant error arising from the loss of other volatile substances (in addition to moisture) which are removed from the acid during the Cenco moisture determination. It was noted further in this study that determinations of moisture levels of less than 1% by the Cenco technique were not generally reliable.

Tablet lubricants are water insoluble materials without pronounced water adsorptive abilities. It is expected that lubricants such as mineral oil, sterotex, and calcium stearate, etc., would show EMC curves similar to those observed above.

Tablet fillers including kaolin, mannitol, terra alba, and dicalcium phosphate had equilibrium moisture contents of less than 1% at all humidities tested. Sucrose was included in this study and, although it evidenced high moisture gain, it did not reach moisture equilibrium at the test humidities during a long storage period (60 days). No reason for this anomalous behavior was apparent. For lactose, the EMC (at the highest humidities) was approximately 2%. This value is higher than that seen with the other fillers and reflects the relative hygroscopicity of this agent.

Extensions of the above considerations to simple formulations can be made. In systems which consist of physical mixtures of two or more agents, each material can seek and reach its own EMC under given storage conditions. The EMC of the mixture will represent a weighted average of the EMC values for the individual components, and predicted EMC values can be obtained for each humidity of interest. Knowledge of the EMC curves for the individual components can be applied in this way to make first estimates regarding the moisture gain of the mixture. This approach also allows a preliminary basis for selection of formula components in line with the desired moisture characteristics of the product.

If no chemical reactions occur during the granulation or tableting of physical mixtures, it can be expected that the EMC curve for these materials will also be related to the EMC values for their individual components. (Changes in particle size of the powders during granulation or compression will effect the absolute values.) The antacid granulation containing high concentrations of relatively hygroscopic materials (sucrose and magnesium trisilicate) therefore would be expected to show higher EMC levels than obtained with the lactose based placebo. Figure 2 indicates that this is true. Similar observations demonstrating the comparative moisture sensitivity of the antacid were made when tablets of both formulations were studied.

EMC values for the placebo granulation were

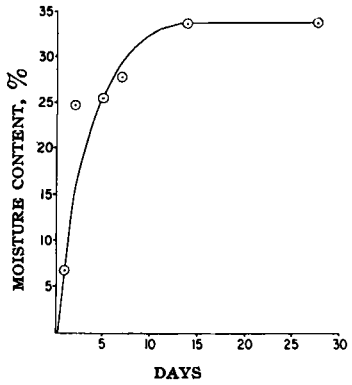

Fig. 6.—Rate of moisture adsorption for antacid granulation at 93% relative humidity.

TABLE I.—STABILITY CHARACTERISTICS OF ASCORBIC ACID TABLETS AT VARIOUS HUMIDITIES

Time, Days		Relative Humidity 93%	81%	73%	56%
3	Assay, %	69	92	100	100
	Moisture gain, %	0.7	0.6	0.0	0.0
5	Assay, %	62	90	97	100
	Moisture gain, %	11	1.4	0.5	0.0
30	Assay, %	29	68	81	84
	Moisture gain, %	46	7.1	1.5	0.1

predicted (from a weighted average of the component EMC values) at 4.6, 3.3, and 2.5% at 93, 83, and 71% R.H., respectively. These predictions are in reasonable agreement with the experimentally determined values of 3.5, 2.8, and 1%. While further studies are required to confirm the quantitative aspects of this approach, these experiments do demonstrate the value of the EMC concept for qualitative predictions of moisture content–humidity interrelationships. It is interesting to note that the use of humectants in cosmetic formulations (16) and flow conditioners in fertilizer products (17) are based on similar considerations.

Stability Considerations.—The static equilibration technique used in the present experiments is similar to that generally employed in following the stability of pharmaceuticals under exaggerated humidity conditions. In such systems, little or no flow of air across the sample is obtained, and mass (moisture) transfer occurs by molecular diffusion alone. Rates of moisture adsorption, therefore, can be expected to be low and prolonged storage times will be required before samples attain their equilibrium moisture content. In the present study it was found that storage intervals of at least 15 days were necessary for samples to achieve equilibrium. This is illustrated in Fig. 6 with representative data for the antacid granulation.

For moisture sensitive materials, significant decomposition can occur in the early storage intervals during which nonequilibrium (moisture) conditions prevail. The rate of decomposition observed during this period should be related, however, to the moisture actually present in (or on) the sample. While the moisture content will be influenced in turn by the storage humidity utilized, simple relationships between relative humidity and decomposition rates may not exist.

These points are illustrated by the data of Table I which gives the assay results and corresponding moisture gain of ascorbic acid tablets stored for various time intervals at four different humidities. At any given humidity, increasing amounts of moisture were adsorbed as the storage time was extended. (EMC levels were not established within a 30-day interval). The amount of moisture adsorbed during any given time period decreased with decreasing humidity. Further, assay values of ascorbic acid were related to the amounts of water adsorbed. Kinetic analysis of the data is difficult, however, since moisture equilibrium was not obtained. Rates of moisture transmission in elevated humidity studies of this type may be, in fact, the overall rate-limiting consideration in the decomposition process.

Rates of moisture gain under exaggerated humidity conditions will be influenced by the physical characteristics of the sample such as its surface area and porosity, etc. For tablets, moisture adsorption rates will be related in part to hardness. Increasing tablet hardness should decrease moisture transfer rates within the solid and thereby decrease the overall rate of moisture gain. This should result, in turn, in improved stability with increasing tablet hardness. To test these predictions, tablets were made from a ferrous sulfate granulation and compressed to hardness of 6, 8, and 10 Strong-Cobb units. After a 3-day storage period at 93% R.H. moisture gain was 8.5, 7.2, and 6.8%, respectively. The decomposition of ferrous sulfate in these tablets was 31.7, 28.3, and 24.1%, respectively, and was in line with the moisture gain values.

Further experiments are required to extend these initial observations. For such kinetic studies, vapor diffusion effects which may limit the overall rates of decomposition will have to be considered. Dynamic techniques for achieving rapid moisture equilibration in the solid will be more useful than the static methods routinely employed in such studies.

CONCLUSIONS

The concept of equilibrium moisture content (EMC) defines the relationship between moisture adsorption and relative humidity. This concept is useful in establishing proper drying and storage conditions for pharmaceutical materials.

Equilibrium moisture contents have been obtained for a number of representative pharmaceutical materials. This data is useful in predicting the effects of storage humidity on the moisture adsorbed by simple physical mixtures. The stability of products containing moisture sensitive active ingredients may be related to their moisture content which in turn is predictable from the EMC curves of individual components.

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Application of Radioisotopes to Absorption in Pharmaceutical Closures

By GLENN S. CLARK and HOWARD A. SWARTZ

Moisture absorption by rubber stoppers was determined by means of tritiated water. Residual activity in the stoppers and ferrules was determined after storage at room temperature at 50° and observed to be 0.56 and 0.025 per cent, respectively, at maximum for time periods involved.

IN THE PACKAGING of biological and injectable pharmaceutical products, the absorption of packaged components into the package materials with a subsequent loss of product is a critical problem. Mattocks and Milosovich (1) reported on the absorption of water by rubber stoppers and on the effects of inorganic salts in solution. Lowry and Kohman (2) discussed the mechanism of transmission into the stoppers and the role of stopper constituents. Barry (3) described the criteria for judging rubber closures in a symposium on the subject. The absorption of bacteriostatic agents by stoppers was reported by Wing (4, 5) and Sykes (6). The measurement of transmission and product loss, with the evaluation of various packaging materials, has been determined essentially by three methods (7). (*a*) Trial and error, using packaging materials of different types and checking weight losses; (*b*) holding the container and contents (sealed) under a vacuum for an extended time and checking the weight loss; (*c*) the Karl Fisher titration method.

These methods are either approximations and establish a series of assumptions or are time-consuming and complex procedure-wise. The time involved in these tests usually extends over a period of several months for a reasonable evaluation. In addition, many variables are involved that are critical or difficult to evaluate.

In the vacuum test method, the weight losses observed are assumed to be due to solvent transmission through or around the stopper and seal. This procedure may accurately determine a loss but neither permits determination of the actual route of solvent transmission, nor allows detection of potential residual uptake of solvent in the stopper involved.

The Karl Fisher titration method has been widely employed for the determination of water loss and absorption; several modifications have been utilized. The complexity of the procedure and necessity of exact details in application to these problems makes it difficult to obtain reproducible data.

In view of these problems and because of the importance of the data to packaging of biologicals, the evaluation of a testing method utilizing radioisotopes was proposed. It was believed that the application of radioisotopes would permit the accurate determination of true absorption, transmission rates, and the actual routes of losses from the containers. The specificity of radioisotopes appeared to lend itself particularly well to these determinations. In conducting the preliminary test it was determined that 15 vials and stoppers, sealed by conventional methods, would serve to check both the theory and the method.

EXPERIMENTAL

Counting Method.—All samples were counted by means of liquid scintillation counting.[1] The instruments were operated at settings determined to permit an efficiency of 40% for tritiated water. All samples were counted for 10 minutes and corrected for background.

Procedure.—Fifteen glass vials[2] of 9-ml. capacity were divided into three groups of five vials each. A 2.5-ml. quantity of tritiated water[3] with a total measured activity of 1.59×10^6 c.p.m. was added to each group. A 20-mm. rubber stopper,[4] preconditioned by autoclave sterilization and siliconed as in production processing, was added to each vial. A 20-mm. aluminum ferrule[5] was placed into position; the vials were sealed with a Firmpress capping device.[6]

One group of five vials was maintained at room temperature for 2 weeks. A second group was maintained at 50° for 1 week in a constant temperature oven, and the third group maintained at 50° for 2 weeks. These periods at 50° are assumed equivalent to 3 months and 6 months at room temperature, respectively (8). At the conclusion of the designated times, the vials were cooled to room temperature, and the ferrules removed. Each ring

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[1] Ekco Liquid Scintillation Detector, model 6641, Ekco Spectrometer and Scaler model N610A, American Tradair Corp., Long Island City, N. Y.
[2] Wheaton Glass Vials 2-104E.
[3] Tritiated Water Reference Standard, Tracer Laboratories Co.
[4] Gillon Stoppers No. A-46.
[5] West Seals No. 20-1.
[6] Firmpress HO-207, Wheaton Co., Millville, N. J.

TABLE I.—PER CENT RESIDUAL ACTIVITY

Vial	Room Temp., 2 wks.		50° C., 1 wk.		50°C., 2 wks.	
	Stopper	Rings	Stopper	Rings	Stopper	Rings
1	0.32	0.019	0.35	0.012	0.53	0.023
2	0.29	0.034	0.41	0.030	0.58	0.009
3	0.36	0.024	0.43	0.026	0.56	0.025
4	0.22	0.007	0.44	0.020	0.50	0.020
5	0.28	0.014	0.33	0.019	0.56	0.025
$\bar{X}$	0.29	0.019	0.39	0.021	0.55	0.020
S_x	0.051	0.001	0.047	0.0068	0.030	0.0067

was placed into a glass counting vial,[7] 1 ml. of absolute ethanol added, and 10 ml. of liquid phosphor introduced. (PPO[8] 4.00%, POPOP[9] 0.05%, and toluene to 1 L.) The vials were labeled by group and numbered.

The rubber stoppers were withdrawn with forceps, dusted with talc, and brushed clean to remove any external adhering water. Each stopper was placed into a glass vial and 10 ml. of ethanol was added. The vial was sealed with a screw cap and incubated at 50° for 48 hours to leach residual water. The 48-hour period was observed to achieve complete extraction of the tritium from the stoppers on the basis of a constant specific activity of the alcohol extract. At the end of this time, the vials were cooled to room temperature, opened, and 1-ml. aliquots measured accurately and added to individual glass counting vials containing 10 ml. of the liquid phosphor solution. Five samples of ferrules and identically treated rubber stoppers were prepared for controls and background measurements.

The activity in c.p.m. for each ferrule and stopper was determined as described and compared to the total activity placed originally into the Wheaton vial to determine the per cent of residual activity. These values are tabulated in Table I and plotted for the stoppers in Fig. 1.

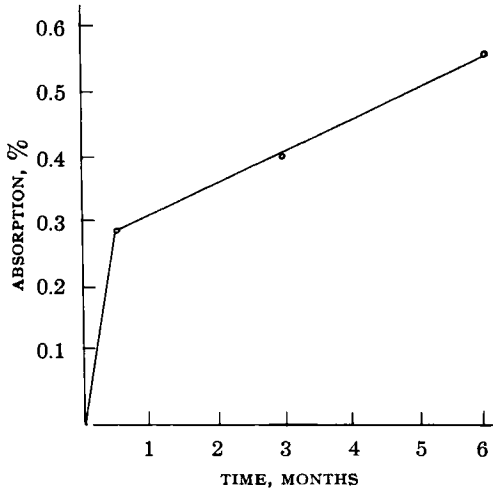

Fig. 1.—The per cent absorption in rubber stoppers *vs.* time in months based on 50°C. incubation periods.

DISCUSSION

The per cent of residual activity detected on both the ferrules and in the stoppers was not the total water uptake or transmission, but only the residue at the conclusion of the incubation period. The rather constant values for the ferrules would indicate that an actual exit passage may occur for the water in the vials, or that this is a saturation level—and once reached, no additional uptake occurs. The uptake of the stoppers, as seen in Table I and Fig. 1, occurs very rapidly and probably commences immediately after sealing the vials. The process is noted to increase in a near linear proportion, reaching the observed peak of 0.55% at the assimilated 6-month period. Whether transmission occurred was not determined, but weighing the sealed vials prior to and at the completion of the incubation periods would serve to measure this factor. This preliminary approach has clearly indicated that uptake into the rubber stoppers does occur, and that a potential exit from the sealed vials may occur around the metal rings. The method is efficient, accurate, and most specific. Any radioactivity detected in the stoppers and on the metal rings could have come only from the tritiated water added to the vials. It should be noted also, that these residues represent a loss of available volume contained within the sealed vial itself, and when an evaluation of solvent loss is made by weighing, this loss would not be detected as it would contribute to total weight.

[7] Counting Vials, American Tradair Corp., Long Island City, N. Y.
[8] PPO-2,5-Diphenyloxazole.
[9] POPOP—*p*-bis [2-(Phenyloxazol 1)]1-benzene.

SUMMARY

The results of the preliminary study indicate that the use of radioisotopes in evaluating vial closures and stoppers and sealing efficiency offers a very specific and rapid method to obtain information with considerable importance to the packaging of biological products.

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Ascorbic Acid Stability in Certain Aqueous and Fruit Juice Vehicles Subjected to Elevated Temperature

By M. C. UPRETY, B. REVIS, and S. M. JAFAR

The effect of heating ascorbic acid in fruit juices for 6 hours in a boiling water bath in the pH range 2.0 to 8.0 was investigated. Pure lime juice, lime juice plus sorbitol (1:1), and lime juice plus glycerol (1:1) were found to be good vehicles. At pH 3.0 the retention was fairly good. Under controlled conditions there was no significant loss even in distilled water. Storage of vitamin C in distilled water at room temperature showed a rapid loss, except at pH 3.0 where the retention was 44 per cent.

IT IS WELL KNOWN that liquid vitamin mixtures gradually deteriorate in their biological potency during storage and during the process of heating. The loss in vitamin potency is dependent on many factors. Various workers have investigated the problem of stability, yet much remains to be done in this direction. Vehicles (1–3) greatly influence the stability of vitamins; an ideal vehicle which may be acceptable to both children and adults has been the subject of long search. With this idea in mind, the present study was undertaken employing fruit juices as vehicles. Fruit juices are preferred because they are natural and palatable. The various formulations used are listed in Table I. Ascorbic acid was subjected to heat in a boiling water bath in the formulations as given in Table I for 6 hours. On the basis of the retention values of ascorbic acid, the suitable vehicles offering maximum protection were employed for further storage studies.

EXPERIMENTAL

Vitamin.—Ascorbic acid (Hoffmann-La Roche and Co., Ltd., Basle, Switzerland) was used.

Vehicles.—Fruit juices, syrup (67% w/v), sorbitol (E. Merck 70% w/v), and glycerol (BDH 75% v/v) were employed. Some of these bases contained 0.15% potassium metabisulfite (E. Merck, henceforth referred to as KMS) and 0.15% cysteine hydrochloride (E. Merck) Table I.

Procedure.—A 5.0 mg./ml. quantity of ascorbic acid was added to the base and the solution was filled in Pyrex test tubes. The pH of all these vehicles was adjusted between 2.0 to 8.0. After recording the initial readings, the test tubes were plugged with cotton and kept in boiling water for 6 hours. Ascorbic acid was estimated by titrating it against standard 2,6-dichlorophenol-indophenol (BDH) solution.

The percentage retention of ascorbic acid after 6 hours of boiling is given in Table I. Another study was made to see the effect of storage on ascorbic acid in glass distilled water at room temperature for a period of 30 days, the results are presented in Table II.

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RESULTS AND DISCUSSION

It is clear from Table II that in distilled water vitamin C was lost to the extent of 70% in all the pH ranges between 2.0 to 8.0, except at pH 3.0 where there was 44% retention. Between pH range 4.0 to 6.0, the destruction was to the same extent, indicating that pH values 4.0, 5.0, and 6.0 behave alike as far as retention of vitamin C in distilled water is concerned.

A slight drop in pH occurred in the sample originally adjusted to pH 8.0. No changes were observed in other sets having a pH 2.0 to 7.0.

At pH 2.0, 7.0, and 8.0 the retention is practically the same. Table II suggests that vitamin C can be stabilized at pH 3.0 in distilled water with the inclusion of some antioxidants and metal binders. Experiments are in progress to stabilize the solution of ascorbic acid at pH 3.0 in distilled water.

At the end of the experimental period (30 days), browning occured at all the pH values under trial. The intensity of the browning was more with corresponding higher pH values. At pH 3.0, browning was comparatively less. It is assumed that the oxidation products of vitamin C were responsible for this browning and probably also because of polymerization of ascorbic acid to furfural. Further work on this aspect is in progress.

It is evident from Table I that even in distilled water ascorbic acid was retained to the extent of 91% at all the pH values, except at pH 4.0 and 7.0, where the retention was 78.0% and 61%, respectively. Hence, it can be concluded that heating does not completely destroy ascorbic acid under controlled conditions of pH in distilled water. This may be explained on the presumption that chances of metallic ion contamination in distilled water are less.

KMS has a stabilizing tendency in the retention of ascorbic acid at all pH values except at pH 2.0. Loss of ascorbic acid in distilled water at pH 4.0 and 7.0 after 6 hours of heating can be counteracted by the addition of KMS at the 0.15% level. Cysteine hydrochloride also has a stabilizing effect on vitamin C at all pH values except pH 2.0.

Lime juice without preservative can retain ascorbic acid to the extent of 100% at pH values 3.0, 6.0, 7.0, and 8.0. At pH values 2.0, 4.0, and 5.0 a slight destruction occurred. Preservative seems to have no influence on the stability of ascorbic acid in lime juice at pH 6.0, 7.0, and 8.0; but at pH

TABLE I.—PERCENTAGE RETENTION OF ASCORBIC ACID AFTER 6 HOURS IN BOILING WATER

Soln. No.	Vehicle	pH 2	3	4	5	6	7	8
1	Water without preservative	97.3	94.4	78.3	91.8	91.8	61.2	91.8
2	Water with preservative	94.4	97.3	91.8	100.0	100.0	100.0	97.2
3	Water with cysteine hydrochloride	94.4	102.8	89.3	94.0	102.8	97.3	102.8
4	Lime[a] juice with preservative	98.0	93.3	89.4	98.5	100.0	100.0	100.0
5	Lime juice without preservative	89.9	100.0	84.9	93.1	100.0	100.0	100.0
6	Lime juice (50%) plus 50% sorbitol	100.0	100.0	91.6	91.6	93.0	100.0	100.0
7	Lime juice (50%) plus 50% glycerol	92.1	100.0	100.0	96.2	97.2	100.0	97.2
8	Lime juice (50%) plus 50% syrup	94.4	88.4	94.4	88.3	97.1	97.2	97.3
9	Sorbitol, 70%	92.8	100.0	91.7	96.5	100.0	100.0	100.0
10	Syrup, 67%	50.0	92.9	77.3	95.4	97.4	98.5	100.0
11	Glycerol, 75%	74.3	80.3	95.8	83.3	90.0	91.9	94.4
12	Mango[e] pulp (50%) plus 50% water with preservative	67.7	98.8	89.3	80.5	82.6	74.3	81.7
13	Mango pulp (50%) plus 50% water without preservative	62.5	74.3	64.4	90.1	94.7	97.3	91.6
14	Pure apple juice[b]	100.0	98.5	88.9	93.2	100.0	93.9	78.0
15	Pure mosambi juice[c]	89.9	89.5	89.5	86.5	92.9	95.2	97.4
16	Pure pineapple juice[d]	95.3	100.6	80.1	95.2	100.0	95.3	95.3

[a] Lime—*Citrus aurantifolia* Swingle. [b] Apple—*Prunus malus*. [c] Mosambi—A variety of *Citrus sinensis osbeck* (sweet orange), grown in Western India. [d] Pineapple—*Ananas sativa* schutt. [e] Mango—*Mangifera indica* L.

TABLE II.—PERCENTAGE RETENTION OF ASCORBIC ACID DURING STORAGE AT ROOM TEMPERATURE FOR 30 DAYS

Soln. No.	Vehicle	pH 2	3	4	5	6	7	8
1	Distilled water	9.2	44.5	27.3	25.3	25.9	9.8	13.3

3.0 preservative seems to have a destructive effect on the stability of ascorbic acid in lime juice. At pH 2.0 and 5.0, preservative seems to have a stabilizing action. Experiments are in progress to confirm this postulation.

In 50% lime juice plus 50% sorbitol at pH values 2.0, 3.0, 7.0, and 8.0 there was no loss of ascorbic acid. At pH values 4.0, 5.0, and 6.0 the loss was to the extent of 10%.

In 50% lime juice plus 50% glycerol vitamin C, retention was 100% at pH values 3.0, 4.0, and 7.0, while at other pH values the loss was not more than 8.0%.

The combination of 50% lime juice plus 50% syrup was a poor vehicle compared to vehicles No. 6 and 7 (see Table I).

In 70% sorbitol there was no loss of vitamin C after 6 hours of boiling, at pH 3.0, 6.0, 7.0, and 8.0. A slight loss was observed at pH 2.0, 4.0, and 5.0.

Sixty-seven per cent syrup and 75% glycerol do not prove to be good vehicles compared to 70% sorbitol. At pH 2.0 in 67% syrup, there was loss of 50% of vitamin C after 6 hours of boiling. Sorbitol proved to be a good vehicle. This finding to some extent is in conformity with Gerber, *et al.* (4), who showed that in 70% sorbitol and in presence of vitamin B_{12} and ferrous gluconate the degradation of vitamin C can be checked.

Vitamin C deteriorated in 50% mango pulp plus 50% water vehicle at all pH values, both in the presence and absence of KMS. It is interesting to note that 0.15% KMS has a stabilizing effect on the retention of vitamin C after 6 hours of boiling at pH 2.0, 3.0, and 4.0; but the same substance has a deleterious effect at higher pH values, *viz.* 5.0, 6.0, 7.0, and 8.0. This finding is being subjected to repeated trials.

There was no significant loss of vitamin C in both apple juice and pure pineapple juice after 6 hours of boiling at all pH values except pH 4.0. At pH 8.0 there was 20% loss of ascorbic acid in apple juice but none in pineapple juice. Mosambi juice is not a suitable vehicle compared to apple and pineapple juice.

For retention of ascorbic acid, pH 3.0, 6, and 7 were found suitable. pH 4.0 appeared to be on the border line for retention values of ascorbic acid after 6 hours of boiling in all the vehicles tried except vehicles No. 7, 8, 11, and 15. Fifty per cent lime juice plus 50% sorbitol was found to be most suitable for ascorbic acid retention, both from the palatability and economic points of views, although 50% lime juice plus 50% glycerol was better. Apparently there is not much difference between bases No. 6 and 7.

After 6 hours of boiling no change in pH values was observed in the samples previously adjusted to pH value 2.0 and 7.0, but a slight drop in the pH was observed in the test tube originally adjusted to pH 8.0. The result of this investigation indicates that the loss sustained by ascorbic acid during heating for 6 hours in different media is not considerable.

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Effect of Enzyme Inhibitors on Acetylcholine-Induced Atrial Arrhythmias

By P. G. ROY†

An investigation was made of the effects of enzyme inhibitors on the acetylcholine-induced atrial arrhythmias. It was found that an interference with normal metabolic processes abolishes the electrical activity of arrhythmic atria. Azide, cyanide, and iodoacetate were the most effective in causing the disappearance of all potentials. Arsenate, fluoride, fluoroacetate, and malonate were considerably less active, while pyrophosphate was least efficacious. It is felt that the inhibition of the Na^+ pump may play an important role in the consistent conversion from the induced arrhythmias to quiescence.

THE REPORT OF CHANG (1), in which iodoacetate was shown to inhibit aerobic as well as anaerobic activity of the isolated rabbit auricle led the way to numerous investigations of enzyme inhibitors on mechanical and electrical atrial parameters (2–10). In keeping with this, Webb and Hollander (11) observed that several enzyme inhibitors produced changes in the membrane electrical properties of rat atrial cells—one of the most characteristic is a shortening of the action potential duration. Since a connection between the amount of energy available, the duration of the action potential, the ionic movements, and the initiation of arrhythmia has been suggested (12, 13), the present work was an attempt to investigate the influence of an interference with normal metabolic processes on the acetylcholine-induced atrial arrhythmias.

EXPERIMENTAL

The methods used for the dissection of rabbit atria, the induction, and the recording of arrhythmias have been described previously (14). Two modifications in the technique need to be pointed out: (*a*) the use of a larger bath containing 150 ml. of Ringer's solution, (*b*) the atria were permitted to recover a regular rhythm in normal Ringer's solution for 90 minutes. The experiments to be described are of two types:

1. Control Acetylcholine (ACh)—induced atrial arrhythmias, the duration of which was noted on surface electrograms until spontaneous arrest.

2. A series of experiments consisting in inducing first the arrhythmias with ACh. On cessation of stimulation, a 5-minute period of arrhythmia was always recorded before the addition of the enzyme inhibitors to the bathing solution. At the end of that time, 1 ml. of their solutions (as sodium salts) was added to make the final concentrations (11) listed in Table I. A reliable criterion of the inhibitory activity was the disappearance of all potentials appearing as quiescence and observed for at least 5 minutes on the surface electrograms. Thereafter, the inhibitor was washed out and (except with iodoacetate and fluoroacetate) the tendency of atria to return to their normal rate and amplitude was usually rapid (10–20 minutes).

RESULTS

Duration of Control ACh-Induced Atrial Arrhythmias.—A summary of these studies is presented in Table I. Of 11 atria, eight developed stable arrhythmias lasting 80 minutes or more, while only three stopped spontaneously within 80 minutes. The period of fibrillation varied from 24 to 155 minutes with a mean duration of 82 minutes. This is not too surprising since the production and maintenance of atrial arrhythmias are dependent upon numerous factors (12, 13).

Enzyme Inhibitors and Atrial Arrhythmias.—The effect of these substances on the ACh-induced arrhythmias is interesting. The data presented in Table I illustrate clearly the capacity of these inhibitors to abolish the electrical activity of arrhythmic atria. It is quite obvious that a quiescence ensues after an interference with the normal metabolic processes of the atrial cells.

Furthermore, there seems to exist a certain relationship between the inhibitory potency and the time required for complete quiescence. Powerful inhibitors, such as azide, cyanide, and iodoacetate proved to be most effective since the disappearance of all potentials was very rapid. As for arsenate, fluoroacetate, fluoride, and malonate—they occupied an

TABLE I.—EFFECT OF ENZYME INHIBITORS ON THE ACh-INDUCED ATRIAL ARRHYTHMIAS

Trials, No.	Inhibitor	Mean Time Required for Complete Quiescence, min. ± S.E.
11	...	82[a] ± 10.9
10	Azide (1 m*M*)	9 ± 1.9
10	Cyanide (0.5 m*M*)	3 ± 0.2
10	Iodoacetate (1 m*M*)	11 ± 0.5
10	Arsenate (20 m*M*)	34 ± 4.6
10	Fluoride (0.25 m*M*)	28 ± 4.6
10	Fluoroacetate (4 m*M*)	32 ± 4.2
10	Malonate (15 m*M*)	38 ± 6.6
10	Pyrophosphate (1.5 m*M*)	69 ± 9.7

[a] Mean duration of control ACh (3.5×10^{-3} *M*)-induced atrial arrhythmias.

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intermediary position, being less active than the former, but more active than pyrophosphate (the least efficacious). Apparently, the lack of activity of pyrophosphate may be associated with the fact that pyrophosphate combines with Ca^{++} and that the walls of the tissue bath slowly became covered with a white layer following its addition. It is not unlikely that the decrease of ionized Ca^{++} neutralized the inhibitory effect of pyrophosphate. The finding that a lack of Ca^{++} in the medium increases the incidence of arrhythmias which tend to be of longer duration supported this suggestion (15).

DISCUSSION

Previous investigations (16) suggested that a mechanism is present in cardiac tissue which actively delays repolarization under normal conditions; this mechanism is maintained by metabolic processes and seemingly is inactivated by ACh. Then, Burn (17) postulated that the onset of atrial fibrillation appears to be linked to the diminution in membrane resistance, to the increased permeability to K^+, and to the increased rate of loss of K^+ which ACh causes. This was extended and confirmed by subsequent works of Klein and Holland (18) and Holland and Briggs (19). They showed that the onset of atrial fibrillation is accompanied by a marked increase in the inward movement of Na^+ and K^+ leaving the tissue in exchange. More recent studies (20–22) established a net increased Cl^- influx during fibrillation that may play an important role in inactivating the marked increase in the inward movement of Na^+. The thesis was developed that maintenance of fibrillation depends upon the rate of entry of Cl^- into a thin barrier, probably the cell membrane, which is the seat of chemical processes driving Na^+ out of the cell (23). According to Woodbury (24), this process whereby the cell continuously uses metabolic energy to maintain an efflux of Na^+ is called active Na^+ transport; the detailed mechanism for utilization of metabolic energy to carry Na^+ out of the cell is not yet known. Nonetheless, Hodgkin and Keynes (25) reported very interesting findings relative to the effect of enzyme inhibitors on the active Na^+ transport. It is beyond the scope of this paper to summarize them all and we shall mention only three: (*a*) Na^+ efflux is decreased to values near zero by the addition, at appropriate concentrations, of a metabolic inhibitor to the bathing fluid. (*b*) K^+ influx is greatly reduced by metabolic inhibitors. (*c*) Na^+ influx and K^+ efflux are not greatly affected by metabolic inhibitors. These observations are well in agreement with those of Leaf (26) who concluded that inhibition of metabolism slows or stops the ion pump so that the Na^+ entering the cell by diffusion cannot be extruded and the K^+ lost from the cell cannot be replaced. The cell membrane potential diminished and Cl^- enters the cell. This requires further entry of Na^+ into the cell to preserve electric neutrality.

Since experimental evidence is increasing that the maintenance of auricular arrhythmias is associated with active ionic movements which are metabolic energy dependent and that the utilization pathways of this energy essential to the Na^+–K^+ exchange seem very sensitive to enzyme inhibitors, it is logical to assume that the inhibition of the Na^+ pump might probably be responsible for the conversion from the induced arrhythmias to quiescence. Indeed, it will appear that if an arrhythmia is to continue, the active Na^+ transport, which is an energy requiring reaction, need not be impaired. When the metabolism is inhibited, the energy supply is cut off through a reduction in the amount of energy available, resulting in a deficient Na^+ pump. The pump being deprived of its source of energy, the Na^+ efflux and the K^+ influx are greatly reduced. The Na^+ will accumulate inside and the K^+ outside the cell. The ionic desequilibrium will tend to lengthen the refractory period usually indicated by a lengthening of the action potential and to delay the return of excitability. Consequently, an atrial fiber which has contracted remains inexcitable for a long time. When excitation spreads to it from a neighboring fiber which is out of phase, the excitation has no result. Thus the arrhythmia stops and quiescence ensues.

In conclusion, it is emphasized that because of the lack of specificity of most enzyme inhibitors, the data presented here do not exclude the possibility that the inactivation of particular enzymes is involved in stopping the electrical activity of arrhythmic atria; they do suggest, however, that the inhibition of the Na^+ pump may be the main contributing factor.

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Some Low Boiling Constituents of Peppermint Oil

By WALTER C. McCARTHY, K. VENKATRAMANA BHAT, CHARLES W. ROSCOE, and LOUIS FISCHER

Isobutyraldehyde and *trans*-2-hexenal (leaf aldehyde) have been identified among the low boiling constituents of peppermint oil.

THE IDENTIFICATION of trace low-boiling constituents of peppermint oil has been studied using a forerun fraction normally discarded in the commercial rectification of peppermint oil.[1] It is necessary to remove this small forerun to improve the taste and odor of the finished oil and to comply with the U.S.P. requirement that the dimethyl sulfide be removed. This forerun represents approximately one-tenth of 1% of the throughput of peppermint oil in the distillery and the oil originates from peppermint grown in the states of Washington and Oregon. This forerun is known to contain dimethyl sulfide, a complex mixture of aliphatic aldehydes and alcohols and monoterpene hydrocarbons.

In this study, isobutyraldehyde has been identified in the fraction which distilled at 60–64°, and *trans*-2-hexenal (leaf aldehyde), a common constituent of volatile oils derived from green leaf sources, was identified in the fraction which distilled at 138–142°. Polarimetric data has indicated the presence of (+)-α-methylbutyraldehyde in the fraction which distilled at 91°.

The presence of isovaleraldehyde, previously reported as a constituent of peppermint oil (1), has been confirmed in the fraction collected at 91°. From the optical rotation of this fraction, +3.7° in a 1-dm. tube, one might conclude that it contains about 80% isovaleraldehyde and 20% (+)-α-methylbutyraldehyde.[2]

Thus, the ratio of these two isomers appears to be similar to the corresponding isoamyl alcohol: active-amyl alcohol ratio in fusel oil. Ehrlich (2) has reported that samples of commercial valeraldehyde made by oxidation of fusel oil showed rotations of +2.82° and +3.37°, measured in 1-dm. tubes.

EXPERIMENTAL

The peppermint oil forerun was first fractionated by distillation through a column 20 mm. in inside diameter and 20 ft. high, packed with single turn glass helices, 1/8 in. in diameter.

The fraction which boiled at 60–64°, n_D^{25} 1.3775, was identified as isobutyraldehyde by conversion to its 2,4-dinitrophenylhydrazone derivative, m.p. 183–184°, no depression upon admixture with an authentic specimen.

The fraction which distilled at a constant 91°, n_D^{20} 1.3930, α_D + 3.7°, was shown to contain the previously recorded (1) isovaleraldehyde by conversion to its 2,4-dinitrophenylhydrazone derivative, m.p. 120-121°, no depression upon admixture with an authentic specimen.

The fraction which boiled at 138–142° was shown to contain *trans*-2-hexenal by the preparation of three crystalline derivatives.

The 2,4-dinitrophenylhydrazone derivative, red needles, melted at 146–147°.

Anal.—Calcd. for $C_{12}H_{14}N_4O_4$: C, 51.79; H, 5.07; N, 20.14. Found: C, 51.66; H, 4.80; N, 20.38. This derivative of 2-hexenal is reported to melt at 144° (3), 145 to 145.4° (4), and 147° (5).

The semicarbazone derivative melted at 175°. This derivative of 2-hexenal is reported to melt at 173° (3, 5), 175–176° (6), and 176.4 to 177.2° (4).

The acid (obtained by alkaline silver oxide oxidation of the aldehyde) melted at 32 to 32.5°, after recrystallization from petroleum ether and from dilute alcohol.

Anal.—Calcd. for $C_6H_{10}O_2$: C, 63.13; H, 8.83. Found: C, 63.23; H, 8.84. The melting point of 2-hexenoic acid has been reported as 32° (7), 32–33° (6), and 33° (8).

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[1] The authors thank Mr. Paul A. Tornow, I. P. Callison and Sons, Chehalis, Wash., for providing several gallons of this forerun for us.

[2] Attempts to separate this fraction into two components by fractional distillation were not successful. The work reported herein was done in 1956–1959, before preparative gas chromatography was generally available. This technique would likely be a valuable method today for the separation of such valeraldehyde isomers in sufficient quantities for further investigation.

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Determination of Camphor and Menthol in Pharmaceutical Products by Gas Chromatography

By KHALID S. BAHJAT†

A simple, reliable method for the quantitative determination of camphor and menthol in pharmaceutical preparations has not been available until now. A practical gas chromatography method has been developed for the simultaneous quantitative determination of camphor and menthol in pharmaceutical preparations, using a flame ionization detector, Apiezon L column, and an internal standard. The method was successfully applied to both cream and liquid preparations. It is a simple, rapid, and reliable method with an accuracy of ±5 per cent.

SINCE many pharmaceutical preparations contain camphor and menthol, it is desirable to use a method which is both fast and accurate for the simultaneous determination of camphor and menthol. The presently used methods, described in the "Official Methods of Analysis of the Association of Official Agricultural Chemists" (1) are time consuming, requiring at least 4.5 hours for camphor and 2 hours for menthol.

The successful separation of menthol-menthone stereoisomers (2–5) and camphor (6) by gas chromatography suggests that it might be used for the simultaneous determination of camphor and menthol in pharmaceutical preparations. Preliminary work showed that the method is simple and rapid. Also it is possible to determine camphor and menthol at concentrations as little as 0.1%.

EXPERIMENTAL

Apparatus.—F and M model 609 flame ionization gas chromatograph[1] equipped with Disc chart integrator.[2] Nitrogen was the carrier gas. Flow rates were measured by the instrument flow meters. The carrier gas pressure was regulated to approximately 30 psig to maintain a flow rate of about 50 ml. per minute. The air flow rate was 365 ml. per minute, while the hydrogen flow rate was 47 ml. per minute. The column was stainless steel, 8 ft. long, with 0.25 in. O.D. The Hamilton 10-μl. syringe,[3] with Chaney adaptor, was used for sample injection.

Column Packing.—In the search for a suitable liquid phase, Apiezon L, silicone gum rubber, silicone oil 200, Quadrol-Saib, and Carbowax 20M were examined. Finally, 20% Apiezon L on Chromosorb W was selected, since it resolves camphor and menthol completely and the retention times are short—an important factor when many samples are to be assayed.

Chromatography.—The column was conditioned at 250° for 24 hours with a nitrogen flow rate of 50 ml. per minute. The column was not connected to the detector during this period. When running analyses, the column was maintained at 160°, detector block at 240°, and injection port at 274°. Sample size range was from 2.0 to 6.0 μl.

Calibration.—The internal standard technique introduced by Ray (7) was used since it was found to be the most accurate, ±1% being easily achieved. Calibration curves were obtained by measuring the ratio of the peak areas of camphor to internal stand-

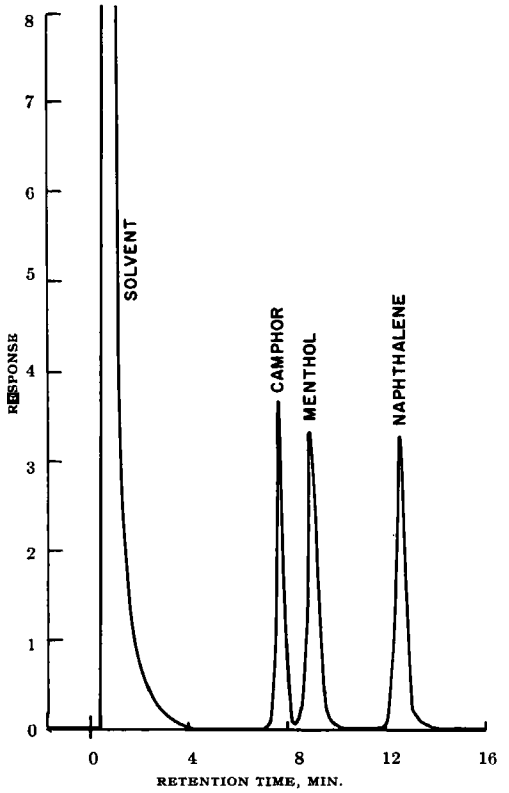

Fig. 1.—Gas chromatogram of an alcoholic solution of camphor, menthol, and naphthalene.

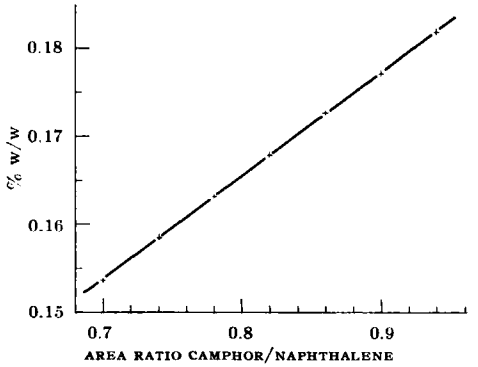

Fig. 2.—Calibration curve for camphor determination.

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[2] Disc Instruments, Inc., Santa Ana, Calif.
[3] Hamilton Co., Inc., Whittier, Calif.

TABLE I.—TYPICAL RECOVERY OF CAMPHOR AND MENTHOL

Menthol			Camphor		
Taken, %	Found, %	Recovered, %	Taken, %	Found, %	Recovered, %
0.150[a]	0.150	100.0	0.173	0.176	101.6
0.175[a]	0.160	96.5	0.500	0.480	96.0
0.200[a]	0.192	96.0	0.200	0.199	99.5
0.300[a]	0.303	101.0	0.300	0.303	101.0
0.400[a]	0.400	100.0	0.225	0.230	102.0
0.100	0.101	101.0	0.100	0.097	97.0
0.150	0.143	95.3	0.250	0.247	98.8
0.250	0.242	97.0	0.150	0.152	101.2
0.350	0.357	102.0	0.400	0.392	98.0
0.500	0.485	97.0	0.350	0.364	104.0
		Av. 98.53%			Av. 99.93%
		Range 95.3 to 102.0%			Range 96.0–104.0%

[a] Cream samples (others are liquid samples).

ard, or menthol to internal standard and plotting the ratio against the percentage of camphor or menthol. This provided a straight line plot.

Naphthalene was selected as an internal standard after examining cetyl alcohol, ethylbenzene, naphthylamine, decyl alcohol, dodecyl alcohol, and eicosyl alcohol. Naphthalene was purified by sublimation before it was used in the experiment. Alcohol solutions having known amounts of camphor, menthol, and naphthalene were used to construct the calibration curves.

Sample Preparation.—(*a*) The liquid sample was injected directly into the gas chromatograph after the addition of the internal standard. (*b*) For the cream sample, a known weight of the sample was extracted with a known volume of 3A ethanol which contained a known amount of the internal standard. Then the alcoholic solution was injected in the gas chromatograph.

The amount of naphthalene, added to both the liquid sample and the alcoholic sample, should be the same as the amount added to the synthetic samples used in construction of the calibration curves.

RESULTS AND DISCUSSION

Under the conditions chosen, retention times for camphor, menthol, and naphthalene were 7.6, 8.8, and 12.4 minutes, respectively (Fig. 1).

Standard solutions, containing known concentrations of the component (camphor and menthol) being determined, were prepared; the ratios of the peak area of this material to the internal standard were measured. Calibration curves were then obtained (Figs. 2 and 3).

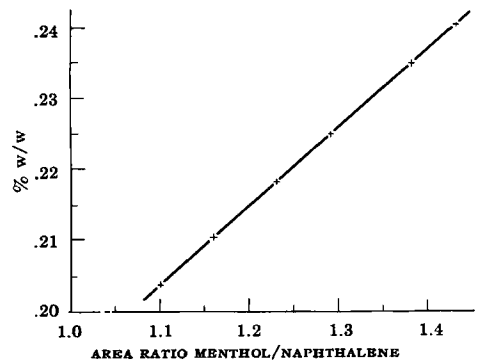

Fig. 3.—Calibration curve for menthol determination.

TABLE II.—PRECISION OF THE METHOD

Menthol		Camphor	
Found, % w/w	Dev. from Mean	Found, % w/w	Dev. from Mean
0.150	+0.003	0.203	+0.003
0.151	+0.004	0.194	−0.006
0.144	−0.003	0.199	−0.001
0.143	−0.004	0.198	−0.002
0.146	−0.001	0.202	+0.002
0.151	+0.004	0.202	+0.002
Av. 0.147		Av. 0.200	

Accuracy.—Samples of cream and liquid preparations were prepared accurately, each with known amounts of camphor and menthol, and were examined under the same conditions as those for camphor and menthol. The results are shown in Table I and are the average of duplicate determinations. The recovery for menthol was 98.53% and for camphor was 99.93%.

Precision.—The precision obtainable with the method applied to duplicate determinations of a synthetic liquid sample is illustrated in Table II. With the camphor, a standard deviation of 1.65% with 95% confidence intervals, ±3.24%, was obtained on six replicate runs, while the menthol results had a standard deviation of 2.45% and 95% confidence intervals, ±4.80%.

SUMMARY

It has been shown that gas chromatography offers a reliable and rapid method for the determination of camphor and menthol simultaneously in cream and liquid pharmaceutical preparations. This assay requires about 30 minutes, and the method is applicable to materials containing concentrations as low as 0.1% (w/w).

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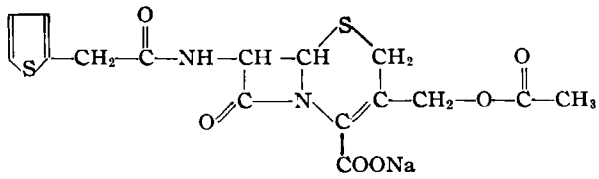
Crystallography of Cephalothin Sodium

By HARRY A. ROSE

CEPHALOTHIN SODIUM is an antibiotic which has shown activity against penicillin resistant organisms. A brief description of the chemistry and microbiological response of the compound has been given elsewhere (1, 2). Data are presented here which permit the identification of this compound by crystallographic methods.

The chemical name of the compound is the sodium salt of 7 (thiophene-2-acetamido) cephalosporanic acid and has the structure



EXPERIMENTAL

This compound can be recrystallized by adding an alcohol to an aqueous solution which results in blades elongated parallel to the c axis. The crystals show the prism 110 and the brachypinacoid 010.

The X-ray powder diffraction data were obtained using copper radiation and nickel filter with a camera 114.6 mm. in diameter. A wavelength value of 1.5418 Å. was used in the calculations. The indexing of the powder pattern was done on the basis of single crystal rotation patterns around both the a and c axes.

TABLE I.—X-RAY DIFFRACTION DATA

Unit cell dimensions: $a_0 = 11.00$ A., $b_0 = 34.20$ Å., $c_0 = 5.05$ Å.
Formula weights per cell: 4
Formula weight: 418.4
Density: 1.477 Gm./ml. (flotation), 1.463 Gm./ml. (X-ray)
Axial ratio: 0.3216:1:0.1477
Space group: $D_2^4 - P2_12_12_1$

TABLE II.—OPTICAL CRYSTALLOGRAPHIC DATA

Refractive indices: $\alpha = 1.568, \beta = 1.592, \gamma = 1.684$
Optic axial angle: $\oplus$ 2V = 56°34′ (calcd. from refractive indices)
Orientation: OAP = 010, $\gamma = a$

TABLE III.—X-RAY POWDER DIFFRACTION DATA

d	I/I_1	hkl	d (Calcd.)
16.52	0.27	020	17.10
10.53	0.50	110	10.47
9.21	0.50	120	9.25
6.71	0.20	140	6.75
5.71	0.07	060	5.70
5.44	0.07	210	5.43
5.20	0.27	220	5.24
5.05	0.27	160	5.06
4.82	0.07	021	4.84
4.49	1.00b	111, 170, 121	4.55, 4.47, 4.43
4.25	0.67	080, 131	4.28, 4.26
4.05	1.00	051, 141	4.06, 4.04
3.78	0.03	061	3.78
3.67	0.07	211	3.70
3.59	0.03	190, 320	3.59, 3.59
3.49	0.50	071, 330	3.51, 3.49
3.36	0.13	340	3.37
3.24	0.20	081	3.26
3.12	0.20	181	3.13
3.01	0.13	111, 0	2.99
2.91	0.20	210, 0	2.90
2.86	0.03	012, 0	2.85
2.78	0.03	341	2.80
2.72	0.13	420	2.72
2.65	0.07	011, 1	2.65
2.61	0.07	440	2.62
2.53	0.13	002	2.53
2.43	0.03	...	...
2.39	0.03	...	...
2.36	0.03	...	...
2.29	0.07	...	...
2.24	0.03	...	...

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Synthesis of 5-Halo-6-methylcinchomeronic Acids

By L. K. GOTTWALD, G. E. McCASLAND, and ARTHUR FURST

New 5-halo-cinchomeronic acids which are structurally related to pyridoxine have been synthesized. These compounds are potential vitamin B_6 antagonists.

IN A CONTINUING SEARCH for antagonists of vitamin B_6 which may be useful as chemotherapeutic agents in tuberculosis or as nonspecific inhibitors of pyridoxine-dependent enzymes, a series of 5-halo derivatives of cinchomeronic acid [3,4-pyridinedicarboxylic acid (I)] were synthesized. These compounds are also useful intermediates for the preparation of substituted nicotinic acids or analogs of pyridoxine (1). An example is the 5-amino-6-methyl derivative II (2–5).

The amino acid II was easily converted by Sandmeyer type reactions to the chloro (III) and bromo (IV) acids in good yields. The iodo acid (V) was obtained by reaction of the diazonium chloride with aqueous potassium iodide. Attempts to prepare the corresponding fluoro acid by the Schiemann reaction, or by diazotization of II in the presence of hydrofluoric acid, were unsuccessful.

The 6-methylcinchomeronic acid VI had previously been prepared by permanganate oxidation (6) of 3-methylisoquinoline and by basic hydrolysis (7) of ethyl 3-cyano-6-methylisonicotinate. We now find that VI is more conveniently prepared from 4-carbomethoxy-3-cyano-6-methyl-2-pyridone (3) by the reaction route VIII → IX → VII → VI. On treatment of the pyridone VIII with phosphorus pentachloride the desired chloro intermediate IX was obtained in good yield. The chloro-diacid VII had previously been prepared by Ryder and Elderfield (8) from the corresponding 3-cyano-4-carboxamide. We find that acidic hydrolysis of the 3-cyano-4-carbomethoxy intermediate IX is equally convenient. Catalytic hydrogenolysis of VII removes the halogen, giving the desired 6-methylcinchomeronic acid VI.

In the course of this work the previously unreported 3-cyano-2,5-dichloro-4-ethoxymethyl-6-methylpyridine (XI) was prepared and characterized.

Biological testing of these compounds is in progress; results will be reported elsewhere.

EXPERIMENTAL

All melting points have been corrected and were measured on a Monoscop or Nalge-Axelrod micro hot stage. Microanalyses by Micro-Tech Laboratories, Skokie, Ill. Infrared spectra were recorded with an Infracord recording infrared spectrometer, using potassium bromide pellets.

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Samples of synthetic starting materials were kindly provided by Dr. W. E. Scott, Hoffmann-LaRoche, Nutley, N. J.
This work was initiated at another institution and was aided in part by Grant CY-2798 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.
A grant to the University of San Francisco by the Eli Lilly Co., Indianapolis, Ind., is gratefully acknowledged.
Abstracted in part from a Master of Science thesis submitted by L. K. Gottwald to the Graduate School, University of San Francisco, 1963.
One of a series of publications on pyridoxine analogs by Arthur Furst and G. E. McCasland (to whom correspondence should be addressed).
Preceding publication: *J. Org. Chem.*, 26, 3541(1961).

5-Chloro-6-methylcinchomeronic Acid Monohydrate (III).—To a well-stirred mixture of 2.15 Gm. of the finely powdered amine (3) (II, m.p. 248°) with 6.0 ml. of 12 *M* hydrochloric acid, kept at from −5 to 0°, was added dropwise over a 10-minute period a solution of 0.9 Gm. of sodium nitrite in 2.5 ml. of water. The mixture became viscous at first but later thinned out. A solution of 0.99 Gm. of cuprous chloride in 1.5 ml. of 12 *M* hydrochloric acid was then added (caution: foaming), and the mixture was allowed to warm to 25°. After heating to 70° for a brief period, the solution was placed in the refrigerator to cool. The white precipitate was collected, washed with water, and dried, giving 1.73 Gm. (74%) of crude product, m.p. 239° dec. A portion of this material was recrystallized from water, giving yellow needles, m.p. 250° with dec. The melting point is dependent on rate of heating.

Anal.—Calcd. for $C_8H_6ClNO_4 \cdot H_2O$: C, 41.14; H, 3.45; Cl, 15.18; N, 6.00; neut. equiv. 117. Found: C, 41.40; H, 3.59; Cl, 15.54; N, 6.00; neut. equiv. 119.

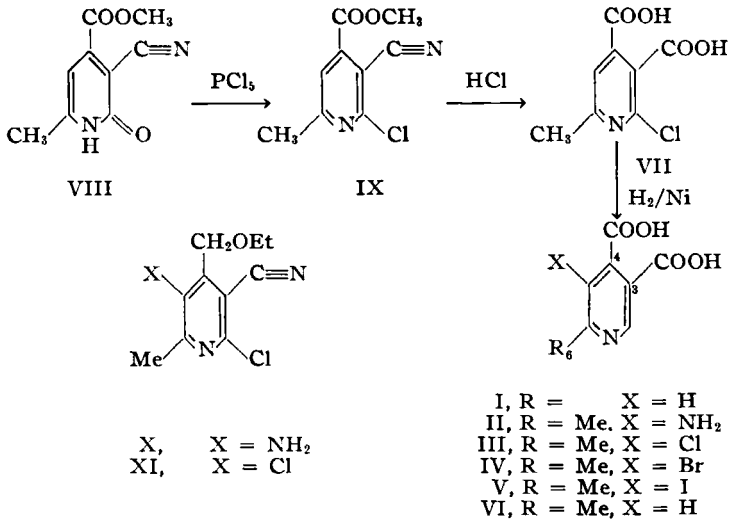
5-Bromo-6-methylcinchomeronic Acid Monohydrate (IV).—The amine (3) (2.15 Gm.) was treated in the manner described above for the chloro analog, but using 8.8 *M* hydrobromic acid, in volumes of 6.0 and 3.0 ml. After final brief heating to 100° and refrigeration, 1.3 Gm. (50%) of the crude product, m.p. 231° dec., was obtained. A portion recrystallized from water gave colorless needles, m.p. 250° with dec. The melting point is almost the same as for the chloro analog and is dependent on rate of heating.

Anal.—Calcd. for $C_8H_6BrNO_4 \cdot H_2O$: C, 34.55; H, 2.90; Br, 28.75; N, 5.04; neut. equiv. 139. Found: C, 34.60; H, 3.02; Br, 28.67; N, 5.11; neut. equiv. 140.

5-Iodo-6-methylcinchomeronic Acid Monohydrate (V).—The amine (3) (2.15 Gm.) was diazotized in the manner described for the chloro analog but using 2.5 ml. of water as solvent for the sodium nitrite. A solution of 1.61 Gm. of potassium iodide in 2.0 ml. of water was added dropwise, to the still cold solution followed by 5.0 ml. additional ice-cold water. The mixture was then allowed to warm to 25°, kept 24 hours, and finally heated to 90–100° for 1 hour. After cooling for several days, the precipitate was collected, washed successively with sodium bisulfite solution and water, and dried, giving 1.51 Gm. (47%) colored crude product, m.p. 240° with dec. A portion recrystallized from water gave tan crystals, melting point unchanged.

Anal.—Calcd. for $C_8H_6INO_4 \cdot H_2O$: C, 29.55; H, 2.48; I, 39.03; N, 4.31. Found: C, 29.81; H, 2.52; I, 39.27; N, 4.24.

The infrared spectra of the chloro, bromo, and iodo acids (III, IV, and V) were very similar, except for small variations in the fingerprint region. The spectra showed O—H stretching absorption at 3500, O—H bending at 1290 and 1263, and carbonyl C=O stretching at 1700 cm.$^{-1}$



I, R = X = H
II, R = Me, X = NH_2
III, R = Me, X = Cl
IV, R = Me, X = Br
V, R = Me, X = I
VI, R = Me, X = H

X, X = NH_2
XI, X = Cl

Methyl 2-Chloro-3-cyano-6-methylisonicotinate (IX).—One-hundred milliliters of phosphorus oxychloride was added to a mixture of 25 Gm. of the pyridone (3) (VIII, m.p. 233°) and 29 Gm. of phosphorus pentachloride. The mixture was heated (anhydrous conditions) at 75–100° for 90 minutes. Phosphorus oxychloride was removed by evaporation *in vacuo*. The dark brown residue was crushed and poured into 100 ml. of ice water. After brief stirring, the product was collected, washed with water, and dried, giving 25.6 Gm. of crude product, m.p. 112–115°. This material was recrystallized from ligroin (1500 ml.), giving 18.5 Gm. (66%) of yellow needles, m.p. 115–116°. A sample recrystallized for analysis melted sharply at 118.5°.

Anal.—Calcd. for $C_9H_7ClN_2O_2$: C, 51.33; H, 3.35; Cl, 16.83; N, 13.30. Found: C, 51.53; H, 3.32; Cl, 16.60; N, 12.97.

2-Chloro-6-methylcinchomeronic Acid (VII).—One-hundred milliliters of 6 *M* hydrochloric acid was added to 10 Gm. of the cyano-ester (IX). The mixture was heated at 90–100° for 5 hours, then cooled. A 2.5-Gm. quantity of yellow crystals was obtained after refrigeration, m.p. 205° dec. The filtrate was evaporated *in vacuo*, giving a yellow residue. The combined crystals and residue were recrystallized from water, giving 4.59 Gm. (45%) of yellow crystals, m.p. 205° with dec. [reported (8) m.p. 205°].

The infrared spectrum showed strong absorption at 2860, 1700, 1290, 1260, and 690 $cm.^{-1}$

6-Methylcinchomeronic Acid (VI).—A 4.0-Gm. portion of the chloro acid (VII) and 2.3 Gm. of sodium hydroxide were dissolved in 75 ml. of water. The mixture was hydrogenated with Raney nickel catalyst at 3 Atm./25° until theoretical hydrogen uptake was reached (about 4 hours). The mixture was filtered and the filtrate adjusted to pH 3 with 16 *M* nitric acid (about 2.7 ml.). The solution was concentrated *in vacuo* to 20 ml., and refrigerated until crystallization was complete, giving 1.3 Gm. (39%) of colorless product, m.p. 240° dec. A sample was recrystallized from water for analysis, giving colorless crystals, m.p. 250° with dec. (reported (7) m.p. 249–251° dec.).

Anal.—Calcd. for $C_8H_7NO_4$: C, 53.04; H, 3.90; N, 7.73; neut. equiv. 90.5. Found: C, 52.54; H, 3.80; N, 7.54; neut. equiv. 90.0.

The infrared spectrum showed strong absorption at 2450, 1700, 1400, 1260, 1060, 892, and 808 $cm.^{-1}$

3-Cyano-2,5-dichloro-4-ethoxymethyl-6-methylpyridine (XI).—A 2.0-Gm. portion of the monochloro-amine (9)(X) was stirred with 5.0 ml. of 12 *M* hydrochloric acid. A clear solution was at first obtained, but the hydrochloride salt soon precipitated. The mixture was treated with sodium nitrite (0.80 Gm.) and cuprous chloride (0.88 Gm.) in the manner described for the chloro compound (III). After final brief heating to 40°, and refrigeration, 1.3 Gm. (63%) of product was obtained, m.p. 50°. A sample recrystallized from ethanol for analysis melted at 52–53°.

Anal.—Calcd. for $C_{10}H_{10}Cl_2N_2O$: C, 49.00; H, 4.11; Cl, 28.93; N, 11.43. Found: C, 48.86; H, 4.19; Cl, 29.07; N, 11.30.

The infrared spectrum showed strong absorption at 2950, 2300, 1590, 1410, 1240, 1160, 1090, 845, 775, and 665 $cm.^{-1}$

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Interaction Between Polyvinyl Alcohol and Procaine Hydrochloride

By C. BOTRÈ and F. M. RICCIERI

The interaction between procaine hydrochloride and polyvinyl alcohol was studied in terms of a determination of the activity coefficient of chloride ions in aqueous solution by means of membrane electrodes. γ_{Cl^-} shows a discontinuity in solution of procaine hydrochloride as a function of the dilution. This discontinuity is enhanced and shifted toward diluted range when amounts of polyvinyl alcohol are present in solution and also a polyelectrolyte behavior is observed.

INTERACTION of macromolecules with chemicals is of great and increasing interest, particularly in the field of compounds with pharmacological activity. Local anesthetics (8–10), steroidic hormones, barbiturates, penicillins, salicylates, and sulfonamides were studied in connection with their interactions with macromolecules (1–7). In some cases the complex formation between several local anesthetics and macromolecules determines a sharp increase in potentiating both duration and intensity of local anesthetic activity (13).

The results obtained by studying the interaction of procaine hydrochloride (PrHCl) and polyvinyl alcohol (PVA) are reported.

EXPERIMENTAL

Materials.—Procaine hydrochloride reagent analytical grade was supplied by Carlo Erba, S.p.A., Milan, Italy. Polyvinyl alcohol from Polymer Consultants, Ltd., London, England, had a molecular weight of 128,000, according to light scattering measurements carried out in our laboratory. Dialyzer tubing of cellulose was supplied by A. H. Thomas Co., Philadelphia, Pa. All other reagents were analytical grade.

Procedure.—Membrane equilibrium dialysis and potentiometric measurements were carried out at room temperature; viscosity and conductivity measurements were performed in a thermostatic bath at 25°.

The solutions of procaine hydrochloride and procaine hydrochloride–polyvinyl alcohol investigated were brought into equilibrium across a dialyzing membrane. For this purpose, two arrangements were set up: (*a*) conventional bags containing the mixed solution of polymer and drug dipped into a vessel containing the drug solution was shaken from time to time, and (*b*) the dialyzing membrane (of about 55 mm. in diam.) was clamped tightly between the flanges of two half cells with a capacity of approximately 50 ml. each. The solution of procaine and procaine + polymer contained in the two half cells was stirred continuously with two glass rods, squeezed at the tips, and running at the same speed into the solution. Both systems were maintained at room temperature until equilibrium was established. Before and after the experiments, the procaine concentration at the two sides of the membrane was determined by measuring the optical densities at 290 mμ with a Beckman (DU) spectrophotometer.

The activity of chloride ions of the solutions containing procaine hydrochloride was determined with positively charged membranes (Permaplex A20). The apparatus and detailed procedure for these potentiometric determinations have been described elsewhere (11).

Conductometric measurements performed in thermostatic bath at 25° ± 0.01 were recorded with a Philips a.-c. bridge, using a cell that allowed the dilution of the stock solution about 15 times.

RESULTS AND DISCUSSION

The membrane equilibrium dialysis measurements obtained in the study of the interaction with polyvinyl alcohol are shown in Fig. 1. The ordinate represents the ratio between the concentration of drugs at the two sides of the membrane at equilibrium; the variation of concentration of polymer is reported on abscissa. The slope of the straight line obtained clearly indicates that an interaction takes place between drug and polymer chain.

Figure 2 shows the activity coefficient of chloride ions (lower) and the specific conductivity (higher) determined in simple PrHCl solution as a function of the PrHCl concentration.

The simpler and more intuitive explanation of such phenomenon could be the existence of small aggregates of PrHCl molecules in water solution above a certain concentration. In fact, the discontinuity and the trend of the plots are similar to that of laurylsulfate and/or similar colloidal electrolytes in the concentration range where the critical micelle concentration takes place (11).

When PVA is added, the plot below the critical point becomes sharply parallel to the abscissa, and the discontinuity is shifted towards lower concentrations of PrHCl (Fig. 3). This shift is also proportional to the concentration of PVA in solution. The localization of the concentration where the break takes place was determined by conductometric and potentiometric measurements; in both cases the agreement was very good.

Below the critical point, the behavior of the activity coefficient is invariant with the concentration and typical of polyelectrolytes (12), while (as previously mentioned) the trend of the plot in absence of polymer in diluted solution is very similar to that of simple electrolytes.

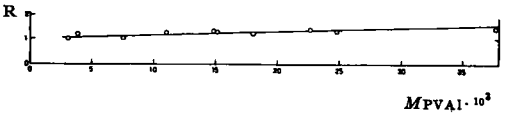

Fig. 1.—Membrane equilibrium dialysis measurements. The ordinate represents the ratio of drug on both sides of the membrane at equilibrium; the abscissa represents the concentration of polymer.

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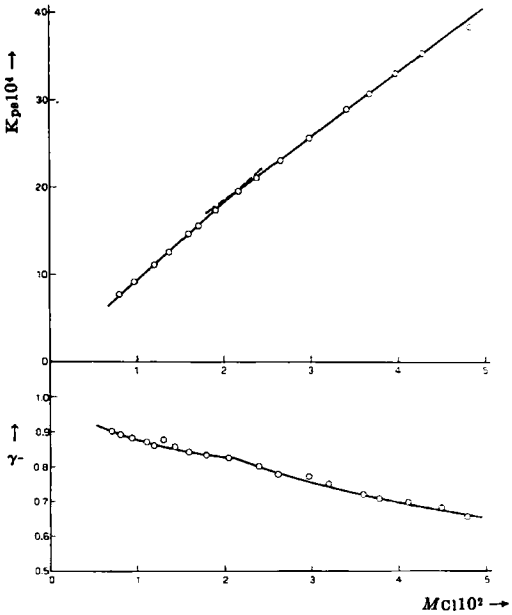

Fig. 2.—The activity coefficient of chloride ions (lower) and the specific conductivity (higher) determined; simple PrHCl solution is shown as a function of PrHCl concentration.

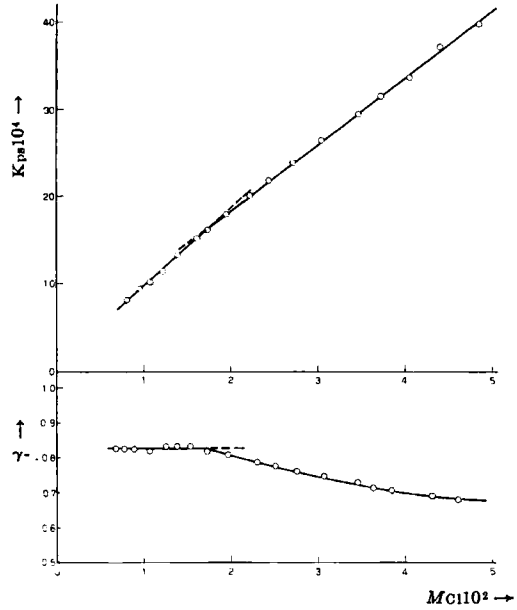

Fig. 3.—Activity coefficient of chloride ions (lower) and specific conductivity (higher) determined in PrHCl solution (PVA also being present) as a function of PrHCl concentration.

In a previous paper (10) Higuchi and co-workers showed that when PrHCl was admixed with polyvinylpyrrilidone (PVP) no interaction takes place. To confirm our potentiometric results, this experiment was reproduced. The determination of activity coefficient of chloride ions with membrane electrodes was perfectly identical to the values of PrHCl in water, thus confirming the lack of interaction.

In conclusion, the results reported here indicate that an interaction takes place between PrHCl and PVA and that the determination of the activity coefficient can be considered a useful method to provide information about interactions between drugs and macromolecules.

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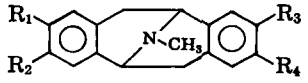
Structure of Bisnorargemonine

Sir:

Bisnorargemonine, $C_{19}H_{21}NO_4$, $[\alpha]_D^{25}$ $-265.8°$ (CH_3OH), is an alkaloid first isolated by Kier and Soine (1) from *Argemone munita* subsp. *rotundata* (Rydb.) G. B. Ownb. It was originally given the trivial designation of *rotundine* which was later changed to *bisnorargemonine* when it became evident that the original name was already in use (2). Bisnorargemonine was found to be closely related to argemonine (2) since it produced the latter on diazomethylation. The structure of argemonine has recently been elucidated by us (3) and independently confirmed by Stermitz and co-workers (4), thus establishing the basic ring structure as that of *N*-methylpavine (I), the structure of which had previously been established by Battersby and Binks (5) on the synthetic compound.



I. R_1, R_2, R_3, R_4, = OCH_3
II. R_1, R_3 = OH; R_2, R_4 = OCH_3
III. R_1, R_2 = OH; R_3, R_4 = OCH_3
IV. R_1, R_4 = OH; R_2, R_3 = OCH_3

As a continuation of our interest in the *Argemone* alkaloids, we have examined the structural possibilities for bisnorargemonine and assign II as the structure based on the evidence cited below.

Bisnorargemonine possesses two phenolic groups and two methoxyl groups. Our previous findings have ruled out a vicinal catechol arrangement of the hydroxyls (2) such as in structure III because 4-ethoxy-5-methoxy-phthalic acid and *N*-methyl-4-ethoxy-5-methoxy-phthalimide have been demonstrated as oxidation products from treatment of diethylbisnorargemonine with potassium permanganate and manganese dioxide-dilute sulfuric acid, respectively. The remaining structural possibilities would be II and IV.

We have utilized the nuclear magnetic resonance spectrum of bisnorargemonine to establish our structural assignment. Considering the two possibilities, it is evident that a decision can readily be made on the basis of the aromatic proton and methoxyl proton absorptions. Structure II has four aromatic protons, all of which differ and, therefore, should be resolvable into four singlets of one proton each. Likewise, the methoxyl groups in II are dissimilar and should show two singlets of three protons each. On the other hand, structure IV has two pairs of identical aromatic protons and two identical methoxyl groups. This should result in two aromatic proton singlets of two protons each and one methoxyl proton singlet of six protons. The nuclear magnetic resonance spectrum of bisnorargemonine[1] showed four nicely separated aromatic proton singlets of one proton each at 3.10, 3.25, 3.34, and 3.44τ and two methoxyl singlets of three protons each at 6.13 and 6.19τ. This establishes the structure of bisnorargemonine as II.

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[1] We are indebted to Dr. M. J. Martell, Jr., and his associates at Lederle Laboratories, Pearl River, N. Y., for the spectrum.

Book Notices

Tropical Therapeutics: Its Pharmacologic Aspects. By ROGER A. LEWIS. Charles C Thomas, Publisher, 301-327 East Lawrence Ave., Springfield, Ill., 1963. x + 276. 14½ × 22½ cm. Price $11.50.

A general pharmacology text is presented which emphasizes drugs likely to be employed in treating diseases in the moist tropics and hot arid zones. Dr. Lewis has had considerable experience in Ghana and India and while he does include general chapters on pharmacology and its relationship and application to drug therapy, the most valuable contribution will be concerning tropical therapy. It seems to be more suitable as a medical student's guide in clinical practice in a tropical area.

Investigation of Rates and Mechanisms of Reactions. Edited by S. L. FRIESS, E. S. LEWIS, and A. WEISSBERGER. John Wiley & Sons, Inc., 605 Third Ave., New York 16, N. Y., 1963. xii + 879 pp. 14½ × 22½ cm. Price $30.00.

Rates and mechanisms of reaction in organic chemistry with the relevant theories which have been presented receive thorough discussion and analysis in this volume in two parts. The work is concerned with the theoretical and experimental tools used to establish the operative mechanisms of reactions. Examples of investigations are given as models for related reactions and where appropriate, comment on possible variations, adaptations, and refinements are included. Part I deals with the kinetic methods and Part II with the nonkinetic methods.

Spices. By JOHN W. PARRY. Chemical Publishing Co., Inc., 212 Fifth Ave., New York, N. Y., 1962. 226 pp. 15.5 × 23.5 cm. Price $8.75.

Clear and simple anatomical descriptions of the external features and internal structures of the various spices are presented in the first part of this work and the chemical composition of spices is presented in the second part. Where pertinent, histological drawings are presented to assist in microscopic examination. For the most part, the material is presented in monograph style.

Paramedical Microbiology. By STANLEY E. WEDBERG. Reinhold Publishing Corp., 430 Park Ave., New York 22, N. Y., 1963. 14½ × 22½ cm. xv + 462 pp. Price $6.75.

A textbook on microbiology of particular value to pharmaceutical educators and pharmacy students is presented. From experiences at the University of Connecticut, Dr. Wedberg has organized the materials for students who have a considerable interest in the application of microbiological principles within their chosen field but less interest in becoming professional microbiologists. Historical background material has been minimized and coverage of nonmedical phases of microbiology is limited to allow greater emphasis on the practical aspects of microbiology in the medically oriented sciences.

Bilharziasis. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. Little, Brown & Co., 34 Beacon Street, Boston, Mass., 1963. xiii + 433 pp. 12½ × 20 cm. Price $11.50.

Twenty-six world authorities present a thorough discussion of modern aspects of research on bilharziasis and its control in this Ciba Foundation Symposium volume. The progress to date is summarized and hopeful lines for future work are delineated.

Practical & Industrial Formulary. By MITCHELL FREEMAN. Chemical Publishing Co., Inc., 212 Fifth Ave., New York, N. Y., 1962. v + 297 pp. 14.5 × 22 cm. Price $7.95.

Hundreds of formulas from cosmetics and perfumes to veterinary preparations are presented in this small cyclopedia intended to provide authentic, concise, and practical information for technical and nontechnical people alike. The great bulk of the book is in recipe form and contains appendices listing suppliers, conversion tables, composition of foods, and other general information helpful to those who utilize the volume.

Intestinal Biopsy. Edited by G. E. W. WOLSTENHOLME and MARGARET P. CAMERON. Little, Brown & Co., 34 Beacon St., Boston, Mass., 1963. 11½ × 18 cm. Price $2.95.

Another Ciba Foundation study group series, this volume presents materials on intestinal biopsy. Topics covered include dissecting microscope appearance of intestinal mucosa, electron microscope study of fat absorption in normal subjects and in patients with idiopathic steatorrhoea, effects of a gluten-free diet on intestinal histology in coeliac disease, significance of mucosal damage, biopsy studies on the pathogenesis of coelica sprue, and intestinal biopsy in tropical sprue.

Pentose Metabolism in Bacteria. By B. L. HORECKER. John Wiley & Sons, 605 Third Ave., New York 16, N. Y., 1963. 11½ × 18 cm. Price $4.00.

A general discussion of the function of the oxidative pathway and the role of pyridine nucleotide coenzymes in the overall energy economy of the cell, and mechanisms for the interconversion of hexoses and pentoses as well as new reductive pathways recently discovered in the filamentous fungi are presented. Additional consideration is given to a comparative study of pentose fermentation mechanisms and their biochemical basis and enzyme mechanisms in aldol and ketol condensations.

Biochemistry. By P. H. GELLINCK. Holt, Rinehart, and Winston, Inc., 383 Madison Ave., New York 17, N. Y., 1963. viii + 308 pp. 14½ × 22½ cm. Price $6.00.

An introductory text book for students with limited backgrounds in organic chemistry and biology is presented. Emphasis has been placed upon the simplicity of the presentation though the more complex formulas and structures are included where necessary. The book appears to be too brief and superficial for most needs of pharmacy students.

Book Notices

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Bilharziasis. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. Little, Brown & Co., 34 Beacon Street, Boston, Mass., 1963. xiii + 433 pp. 12½ × 20 cm. Price $11.50.

Twenty-six world authorities present a thorough discussion of modern aspects of research on bilharziasis and its control in this Ciba Foundation Symposium volume. The progress to date is summarized and hopeful lines for future work are delineated.

Practical & Industrial Formulary. By MITCHELL FREEMAN. Chemical Publishing Co., Inc., 212 Fifth Ave., New York, N. Y., 1962. v + 297 pp. 14.5 × 22 cm. Price $7.95.

Hundreds of formulas from cosmetics and perfumes to veterinary preparations are presented in this small cyclopedia intended to provide authentic, concise, and practical information for technical and nontechnical people alike. The great bulk of the book is in recipe form and contains appendices listing suppliers, conversion tables, composition of foods, and other general information helpful to those who utilize the volume.

Intestinal Biopsy. Edited by G. E. W. WOLSTENHOLME and MARGARET P. CAMERON. Little, Brown & Co., 34 Beacon St., Boston, Mass., 1963. 11½ × 18 cm. Price $2.95.

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Pentose Metabolism in Bacteria. By B. L. HORECKER. John Wiley & Sons, 605 Third Ave., New York 16, N. Y., 1963. 11½ × 18 cm. Price $4.00.

A general discussion of the function of the oxidative pathway and the role of pyridine nucleotide coenzymes in the overall energy economy of the cell, and mechanisms for the interconversion of hexoses and pentoses as well as new reductive pathways recently discovered in the filamentous fungi are presented. Additional consideration is given to a comparative study of pentose fermentation mechanisms and their biochemical basis and enzyme mechanisms in aldol and ketol condensations.

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An introductory text book for students with limited backgrounds in organic chemistry and biology is presented. Emphasis has been placed upon the simplicity of the presentation though the more complex formulas and structures are included where necessary. The book appears to be too brief and superficial for most needs of pharmacy students.

Book Notices

Tropical Therapeutics: Its Pharmacologic Aspects. By ROGER A. LEWIS. Charles C Thomas, Publisher, 301-327 East Lawrence Ave., Springfield, Ill., 1963. x + 276. 14½ × 22½ cm. Price $11.50.

A general pharmacology text is presented which emphasizes drugs likely to be employed in treating diseases in the moist tropics and hot arid zones. Dr. Lewis has had considerable experience in Ghana and India and while he does include general chapters on pharmacology and its relationship and application to drug therapy, the most valuable contribution will be concerning tropical therapy. It seems to be more suitable as a medical student's guide in clinical practice in a tropical area.

Investigation of Rates and Mechanisms of Reactions. Edited by S. L. FRIESS, E. S. LEWIS, and A. WEISSBERGER. John Wiley & Sons, Inc., 605 Third Ave., New York 16, N. Y., 1963. xii + 879 pp. 14½ × 22½ cm. Price $30.00.

Rates and mechanisms of reaction in organic chemistry with the relevant theories which have been presented receive thorough discussion and analysis in this volume in two parts. The work is concerned with the theoretical and experimental tools used to establish the operative mechanisms of reactions. Examples of investigations are given as models for related reactions and where appropriate, comment on possible variations, adaptations, and refinements are included. Part I deals with the kinetic methods and Part II with the nonkinetic methods.

Spices. By JOHN W. PARRY. Chemical Publishing Co., Inc., 212 Fifth Ave., New York, N. Y., 1962. 226 pp. 15.5 × 23.5 cm. Price $8.75.

Clear and simple anatomical descriptions of the external features and internal structures of the various spices are presented in the first part of this work and the chemical composition of spices is presented in the second part. Where pertinent, histological drawings are presented to assist in microscopic examination. For the most part, the material is presented in monograph style.

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The Editor comments

OF WHAT SIGNIFICANCE STATISTICS?

We have been pleased to note that a greater appreciation of the usefulness and value of statistics is gradually evolving in the scientific community. Perhaps the greatest impetus for this awakening comes from the field of clinical testing. By applying proven, reliable statistical methods, test data which on first glance appeared to lead to certain conclusions have been shown to be of highly dubious worth; conversely, in other cases, the opposite sometimes has been true. And more recently, the same or similar mathematical principles have been applied to the disciplines of analytical chemistry, bacteriology, and pharmacology, to name just a few.

The utilization of this valuable tool has been fostered in a number of ways. Many colleges are offering introductory and even advanced courses in statistics to regular day students, and evening lectures to those who wish to further their education while engaged in full-time employment. A number of larger industrial concerns have made arrangements for in-plant lecture courses by qualified instructors so as to provide their employees with the opportunity to study the fundamentals of this subject. And many of our ambitious colleagues, armed with one or two textbooks on statistics, have gained a reasonably good grasp of the principles, solely through home study.

Unfortunately, however, some workers have become overly enthusiastic following their introduction to this science. Like a child with a new toy, they employ it with neither discrimination nor discretion. It is our opinion that if the data obtained from a certain study are too meager to warrant publication, then dressing up the results with mathematical trimmings will not transform the material into a first-rate manuscript. Furthermore, it is not necessary to belabor the obvious. If in an experiment with 50 mice, all 50 died, statistics generally will add nothing to the interpretation.

For the most part, however, the science of statistics has proven to be a very worthwhile aid with much of the work currently in progress both in university and industrial laboratories. We confidently expect that as it becomes better known and understood, it will come to serve us even more, and will take a prominent place among the other indispensible tools of the pharmaceutical scientist. The more prudently statistics are applied, the sooner this day will come.

Edward G. Feldmann

JOURNAL OF Pharmaceutical Sciences

November 1963 volume 52, number 11

Review Article

Column Partition Chromatography in Pharmaceutical Analysis

By JOSEPH LEVINE

PERHAPS THE MOST widely applied separation process used in the analysis of pharmaceutical products is liquid-liquid extraction, wherein a solute is distributed between two immiscible phases. Its concentration in each of the two phases at equilibrium is governed by its distribution coefficient. Partition chromatography may be regarded as an extension of conventional liquid-liquid extraction, in which one of the two liquid phases is present in the form of a film sorbed by particles of an inert support. A very large surface area is required to reach equilibrium within a reasonable time interval, since interphase transfer of solute takes place at the liquid-liquid interface. The large surface area is furnished by the finely divided particles of the support.

The support is variously referred to as the solid phase, the supporting phase, the supporting medium, or the inert phase; the sorbed liquid is known as the immobile phase, the internal phase, the nonmobile phase, or the stationary phase; and the second liquid phase is termed the mobile phase, the developing solvent, or the eluant.

Column chromatography has been defined as "uniform percolation of a fluid through a column of more or less finely divided substance, which selectively retards certain components of the fluid" (1). Cassidy (2) defines partition chromatography as "a method of separating substances by distributing them between two liquid phases, one of which is mobile and the other essentially fixed by sorption to a support." He does not, however, restrict the process to liquid-liquid distribution; he states that "the support itself may or may not be active in the separation process." Catch, *et al.* (3), said that "doubtless the process (occurring on the column) is a combination of 'chemical' chromatography and 'partition' chromatography."

Meinhard (4) attributed to the supporting material a major role in the separation process. According to him, a layer of adsorbed liquid at a solid-liquid interface may extend to a depth of 100 molecules or more if the solid is "wetted" by the liquid. The structure of the adsorbed layer is modified to various degrees by the adsorbent. Therefore the liquid layer does not behave as an independent entity during the chromatographic process; the stationary phase is comprised of the layer of liquid as modified by the adsorbent. Hence the difference between ordinary chromatography and partition chromatography depends primarily on the media. Büchi and Soliva (5), on the other hand, exclude the effect of the support. They state that partition chromatography is not concerned with the equilibrium between adsorbed and dissolved material, but only with an equilibrium of dissolved material in two solvents which are immiscible or of limited miscibility.

The concept of partition chromatography was introduced in 1941 by Martin and Synge (6) during an investigation of the amino acid composition of wool. Initially they used a 40 stage countercurrent extraction apparatus to separate

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the acetylated amino acids (7). In seeking mechanical means to simplify this process, they sorbed the aqueous phase on finely divided silica gel and packed the mixture in a glass tube. By this means, one of the liquid phases was retained in a rigid position, while the second liquid phase simply percolated through it, making contact in passing.

Martin and Synge visualized the chromatographic column as a number of separate and consecutive regions, in each of which equilibrium of the solute is attained between the mobile and immobile phases. Thus each region or "theoretical plate" is equivalent to a separate vessel of a countercurrent extraction apparatus. In practice, they found that a column 20 cm. in length gave a separation equivalent to that of over 1000 theoretical plates.

In their characterization of the column as a series of "theoretical plates," Martin and Synge presented a mathematical analysis of the process which occurs on the column. In a column having a large number of theoretical plates, they let r = the serial number of the plate, Q_r = the quantity of solute in plate r, v = the volume of solvent used in the development of the chromatogram, α = the equilibrium partition coefficient of the solute = concentration in nonmobile phase/concentration in mobile phase, A = area of column, A_s = area of nonmobile phase, A_L = area of mobile phase, h = height of equivalent theoretical plate, $V = h(A_L + \alpha A_s)$, and R = (movement of band of maximum concentration)/(movement of mobile solvent).

Considering the case where a unit mass of a single solute is put onto the first plate and then followed by pure solvent, they constructed a table showing the quantity of solute in each plate after successive infinitesimal volumes of mobile phase have passed through. From this they showed that the quantity of solute in each plate is given by

$$Q_{(r+1)} = \frac{1}{\sqrt{2\pi r}} (v/rV)^r e^{r-v/V}$$

When $v/rV = 1$, $Q_{(r+1)}$ is a maximum and equal to $1/\sqrt{2\pi r}$ so that the position of maximum concentration has moved a distance hv/V directly proportional to the volume of liquid, v, which has passed through.

Now

$$R = \frac{hv/V}{v/A} = \frac{Ah}{V} = \frac{A}{A_L + \alpha A_s}$$

Then the partition coefficient, α, is $A/RA_s - A_L/A_s$. Martin and Synge found that partition coefficients calculated by this formula agreed closely with the values obtained by conventional procedures. They plotted a curve showing the ideal separations attainable by partition chromatography and discussed the factors contributing to deviations from their theoretical values. The principal source of the deviation is adsorption of solute by the supporting material. Among the other causes are diffusion of the mobile phase, which is affected by temperature, rate of flow, and changes in partition coefficient with change in concentration. Interaction of two solutes, with the more strongly adsorbed solute "eluting" the less strongly adsorbed, can result in separations better than theoretical.

Craig (8) did not accept the correlation between liquid-liquid partition ratios and rates of migration of bands on a column to be proof that partition chromatography is a liquid-liquid extraction process. He believed that "at best this evidence is only circumstantial, and probably reflects more the uniform shift in nearly any physical property in a homologous series than the particular property responsible for such an observed effect." He found the results of partition chromatography to be of very little use in selecting systems for countercurrent distribution. He stated that "certainly, the difference between countercurrent distribution and so-called partition chromatography is much greater than that between Tswett (adsorption) chromatography and partition chromatography. In fact there seems little to be gained by assuming that liquid-liquid partition plays any significant role in the latter process." Subsequent developments in partition column techniques support the interpretation of Martin and Synge. Separations based upon the degree of ionic dissociation, as discussed below, are patently liquid-liquid partition phenomena. The contribution of adsorption is subordinate and merely a complicating factor in these separations.

METHODOLOGY

Commercial silica, designated by Martin and Synge (6) as "pure precipitated" silica, was used as the supporting phase in the introductory application of column partition chromatography. Even in the initial publication on this technique, however, it was noted that separate lots of silica vary significantly with respect to their adsorptive power and their capacity for sorption of water. Several procedures have been reported for pre-

paring and standardizing silica gel suitable for use as a support (6, 9–12). Isherwood (13) and Resnik, *et al.* (14), described purification processes designed to decrease the adsorptive properties of the material.

A number of other materials have been used instead of silicic acid. Synge (15) and Elsden and Synge (16) used raw potato starch as the supporting medium for the aqueous phase. Various forms of cellulose have been used in column partition chromatography as well as in paper chromatography. In a note, Gordon, *et al.* (17), referred to the use of cellulose powder. Hough, *et al.* (18), prepared powdered cellulose by rubbing ashless filter tablets through an 80-mesh sieve. Baker, *et al.* (19), and Brindle, *et al.* (20), used Solka-Floc, a commercial powdered cellulose. Mitchell and Haskins (21) used a pile of 40 circles of filter paper as a column. Stoll and Kreis (22) used cotton linters, which are minute fibers of almost pure cellulose. In a discussion of the nature of supporting material, Martin (1) observed that the initial concept of cellulose as a completely inert support for aqueous phase has changed in favor of one considering the hydrated cellulose to be a strong polysaccharide solution or a complex gel. He concluded that the most ideal partition effect is achieved with siliceous earth (kieselguhr, diatomaceous earth, infusorial earth).

The first use of this material as supporting phase was reported by Catch, *et al.* (3), who used Hyflo Super-Cel[1] in the separation of the penicillins. The use of Celite[1] 545, which is now probably the most widely used supporting phase, was introduced by Peterson and Johnson (23). Other grades of Celite (503 and 535) have been used (24, 25). In a number of publications the material is simply termed "kieselguhr," with no further reference to its identity, source, or quality.

Hegedüs, *et al.* (26), washed Hyflo Super-Cel with a series of organic solvents to remove extractable impurities. Carol, *et al.* (27), removed alkaline buffering impurities of Celite 545 by an acid treatment. An acid-washed Celite 545 is now commercially available.

Stoll and Kreis (22) prepared "diatom stone" (diatomitstein) for use as support by calcination of a mixture of kieselguhr and clay. Chilton and Partridge (28) used 60-mesh Pyrex glass powder as support in the separation of the pomegranate alkaloids.

Although siliceous earth has a much lower adsorptive capacity than silicic acid, losses of alkaloid due to adsorption on this support have been reported (28, 29). Modification of the immobile phase (30) or of the mobile phase (31) has been very effective in eliminating this loss by adsorption. Graf (32) used activated alumina as supporting phase, but he recognized that the process occurring on the column was not simple partition chromatography.

Celite 545 is the most uniform in particle size of the siliceous earths commercially available in this country. The particle size of Celite 545 is 20–40 μ; of Celite 535, 10–40 μ; of Celite 503, 6–40 μ; of Hyflo Super-Cel, 2–20 μ (33).

Howard and Martin (34) modified kieselguhr to permit "reverse phase" partition chromatography. They treated Hyflo Super-Cel with dimethyldichlorosilane, thereby attaching hydrophobic alkyl groups to the surface of the particles. Using this modified material, a nonpolar organic solvent which is normally used as mobile phase may be used as immobile phase, while an aqueous solution of a polar solvent becomes the mobile phase. Boldingh (35) used a column of powdered vulcanized rubber, partly swollen by treatment with benzene. The rubber functions as both supporting phase and immobile phase.

Water was used as immobile phase in the initial applications of partition chromatography (6). Catch, *et al.* (3), used a solution of alkali carbonate. Following this, various aqueous solutions were substituted for water as immobile phase. Isherwood (13) used 0.5N sulfuric acid to suppress the ionization of weak organic acids. Peterson and Johnson (23) used 27 N to 35 N sulfuric acid, and Graham (36) used concentrated hydrochloric acid. Ferric chloride solution was used (37) to achieve complexation with phenolic compounds.

Nonaqueous fluids may also be used as immobile phase in partition chromatography, in conjunction with immiscible organic solvents. Among those which have been used are nitromethane (38), propylene glycol (19), formamide (39), absolute ethanol (40), and a mixture of concentrated and fuming sulfuric acid (41).

A solution of the sample itself may be used as immobile phase in the partition column, eliminating a preliminary extraction step. Graf (32) and Schill and Ågren (42) added sufficient supporting phase material directly to an aqueous extract of alkaloid-bearing plant material to sorb the liquid. Baker, *et al.* (19), mixed Solka-Floc with an oil solution of adrenocorticosteroids. Banes (43) extended this mode of sample preparation to drugs in various dosage forms.

The apparatus used in partition chromatog-

[1] Marketed by Johns-Manville Corp.

raphy may be quite simple. It requires only a glass tube to which an outlet tube is attached. Flow of the mobile phase may be regulated by a stopcock or by a screw clamp on rubber tubing attached to the outlet tube. A support is generally placed at the lower end of the column to retain the column packing. This may be a perforated or a sintered disk, either sealed in or fitted, or it may be a simple wad of cotton or glass wool. Although catalogs of most laboratory equipment suppliers list chromatographic columns with stopcocks, disks, and ground glass connections, the simple column without such fittings is not commercially available at this time.

Several procedures have been described for mixing the immobile phase with the support and for packing the column with this mixture. The immobile phase may be added directly to the solid (6) or to a stirred suspension of the supporting phase in immobile solvent (44). In preparing columns using silica gel, Fischbach, *et al.* (11), determined the ratio of immobile phase to support by adding the fluid to the solid in small increments, mixing after each addition until the mixture became sticky and began to "ball." They then used 5 to 10% less immobile phase than this quantity to prepare the column. Haenni, *et al.* (45), found that a ratio of slightly less than 1 ml. of aqueous immobile phase per gram of Celite 545 is about the correct proportion to prepare a pack with suitable physical characteristics. However, satisfactory separations have been reported (37) with a ratio of 7 ml. of aqueous phase to 5 Gm. of Celite 545. Chilton and Partridge (28) found that 7 Gm. of powdered glass was required to support 1 ml. of aqueous phase.

A number of variaitons in the preparation of the columns have been described. Martin and Synge (6) transferred a slurry of the prepared mixture in chloroform to the column in small portions. As the chloroform ran through, the gel, which floated in the chloroform, packed down and did not refloat during addition of further amounts of slurry. Ramsey and Patterson (12) applied compressed air at a definite pressure to pack the mixture, while Bergström and Sjövall (46) applied reduced pressure at the outlet of the column. Many authors stipulate that a head of fluid be maintained above the packing. Graf (32) simply transferred the prepared mixture to the empty column and compressed it by tamping with a glass rod.

For the resolution of compounds which have relatively small differences in partition coefficients, it is essential that the column be carefully and uniformly packed. This high degree of uniformity in the column packing is not necessary for the resolution of mixtures which are separated because of relatively wide differences in their solubility characteristics, their ionic species or their ionic strengths. Nevertheless, many published analytical procedures which are based upon these properties have specified the preparation of the very meticulously packed column, although the same results can be obtained with a very simple, rapidly prepared column. (Compare, *e.g.*, References 47 and 48.)

The rate of elution or development may be controlled in several ways. The mobile phase may flow over the partition column by gravity, with the aid of air pressure above the column, or by connecting the outlet of the column to a suction flask and applying reduced pressure. The rate of flow may be controlled by the amount of pressure or vacuum applied, with a stopcock or a screw-clamp on tubing attached to the outlet of the column, by the height of the head of fluid above the column, or by the density of the column packing. Rates of flow of mobile solvent have been reported over a range of from $1^1/_4$ ml. per hour (26) to 10 ml. per minute (49). Haenni, *et al.* (45), discuss critically the factors involved in determining optimum flow rate. The same authors describe an apparatus for obtaining a highly uniform flow rate throughout the development of the chromatogram (44).

TECHNIQUES

Partition chromatography was first conceived as a form of countercurrent distribution. Most of the early applications of the technique were designed to achieve separations based on relatively small differences in distribution coefficients. It was soon found that processes other than simple countercurrent extraction can be conducted very conveniently and efficiently on the partition chromatographic column. These range in complexity from the isolation of a single substance from its dosage form to the conducting of a chemical reaction on the column in the immobile phase. Frequently the modifications of the partition process were patterned on techniques used in adsorption chromatography, but many were designed specifically for the partition column. In adsorption chromatography, a mixture of materials may be selectively desorbed by changing solvents after the elution of the first component. In partition chromatography, the same process is applied to substances having significantly different partition coefficients. This

procedure was introduced by Evans and Partridge (50), who separated hyoscine and hyoscyamine on a *kieselguhr:pH 6.2 buffer* column,[2] eluting the hyoscine with ether and the hyoscyamine with chloroform. Marvel and Rands (51) used mixed solvents as eluant, increasing the proportion of polar solvent stepwise; Donaldson, *et al.* (52), devised an apparatus for achieving this increase continuously.

The influence of buffers upon the separation of substances which form dissociable salts has been discussed by several investigators. Jensen and Svendsen (53) showed that while strychnine and brucine cannot be separated by elution from a column in which water is immobile phase, they are readily separated if buffers at pH 7 or lower are used.

The relationship between degree of dissociation and rate of elution is derived from the equation (54): $\log \alpha' = \log \alpha + \text{pH} - \text{pK}$, where α' is the partition coefficient of the dissociated compound, α is the partition coefficient of the undissociated compound, and K is the dissociation constant.

Büchi and Soliva (5) point out that the partition coefficient of a weak acid or base in a buffer solution is a simple function of pH. In an extension of this equation to encompass mixtures of bases, they showed that, at a constant pH, the degree of separation which is achieved conforms to

$$\beta = \frac{\alpha' B_1}{\alpha' B_2} = \frac{\alpha B_1}{\alpha B_2} \cdot \frac{K B_2}{K B_1}$$

where β is termed the "separability factor" and B_1 and B_2 are weak bases. Bettschart and Flück (55) treated the subject in a similar manner. Bottomley and Mortimer (56) stated that alkaloids are eluted from a buffered column in order of their $\text{pH}_{1/2}$ values (where the term $\text{pH}_{1/2}$ designates the pH at which $\log \alpha' = 0$; *i.e.*, the compound is distributed equally in the two phases).

Schill and Ågren (42) performed on the partition column the counterdistribution of a base between an organic solvent and aqueous acid or alkali, which constitutes the basis of the classical alkaloidal assays. They used 0.5 *M* H_3PO_4 as immobile phase to trap the alkaloids from ether solution while neutral or acidic components passed through the column. They then transformed the immobile phase to an alkaline pH *in situ* with ammonia-saturated chloroform, which eluted the alkaloid as the liberated free base. Solutions of diethylamine in ether (57) and of triethylamine in chloroform (58) have been used for the same purpose. Bitman and Sykes (25) bubbled gaseous carbon dioxide onto a column containing sodium hydroxide as immobile phase to convert it to sodium bicarbonate.

Carless (57) changed the pH of the buffer used as immobile phase gradually and continuously, progressing from pH 3.0 to pH 4.8 by adding 0.1% pyridine to the developing solvent. He considered this process analogous to gradient elution methods, since a pH gradient existed down the column. To achieve the inverse process, acidification of an alkaline immobile phase *in situ*, a chloroform solution of glacial acetic acid has been used (59).

Multistage columns to separate mixtures of drugs (59) have been reported. Separate stages having acid and alkali as immobile phase were used either as layers in the same column or in separate columns arranged in series (58). These layers "trap" alkaloidal or acidic drugs as their water-soluble salts from solution in the nonaqueous mobile phase. The separated drugs were recovered after reversal of the pH of the immobile phase as described above.

PROCEDURES

Steroids.—Column partition chromatography has been widely used in the separation of the steroid hormones, particularly the estrogenic steroids. Nyc, *et al.* (60), separated estriol, estradiol, and estrone by reverse-phase partition on a rubber column. The stationary phase was powdered rubber swollen in 10% methanol saturated with benzene. A mixture of the estrogens in 20% methanol was passed onto the column; estriol was eluted with this same solvent. Estradiol was removed with 40% methanol, and estrone with 60% methanol. Swyer and Braunsberg (61), using a column of *Celite 535:2.3 N sodium hydroxide*, with benzene as eluant, separated estrone and estradiol in order; estriol remained on the column. Stern and Swyer (62), using the same system, successfully eluted the estriol with chloroform-butanol (3 + 1). Haenni, *et al.* (45), described in detail the optimum conditions for quantitative separation of estrogenic diols. They used Celite 545 as support and 0.4 *N* sodium hydroxide as stationary phase. This concentration of sodium hydroxide was selected as requiring the minimum volume of developing solvent to achieve a desired degree of separation of β-estradiol from α-dihydroequilin. They weighed the importance of temperature, flow rate of eluant, ratio of immobile to mobile phase, and degree of pack-

[2] This terminology will be used to designate the supporting phase: immobile phase combination.

ing of the column as factors in the separation. The effect of temperature is twofold. The partition coefficients of the diols change at different rates with temperature, and the band volume of the individual compound varies inversely with temperature. Two columns were described. The estradiols and dihydroequilins as a group were separated from the dihydroequilenins on one and the estradiols were separated from α-dihydroequilin on the second. They defined the volume of mobile solvent required for the elution of each component, giving the correction of these volumes for variations in temperature. The same authors (44) described the analysis of commercial estrogenic preparations, including oil solutions, tablets, and suspensions. The mixture of α-dihydroequilin and α- and β-estradiol was separated from nonestrogenic impurities, traces of estrone and equilin, and the dihydroequilenins, and the individual components of the mixture were determined by a differential colorimetric procedure. A collaborative study (63) of the combined chromatographic separation and colorimetric procedure was conducted for the determination of the physiologically active estradiol-17β. (At the time of publication the configurations of the estradiols had not been established. It was later shown that the compound then referred to as α-estradiol is actually estradiol-17β.)

Bitman and Sykes (25) separated estrone, estradiol, and estriol in a two-step operation. They eluted estrone and estradiol successively with benzene from a column of sodium hydroxide on Celite, in a procedure analogous to those described above. The more strongly acidic estriol was not eluted under these conditions. After the pH of the immobile phase was lowered by bubbling carbon dioxide over the column, benzene eluted the liberated estriol.

Carol, *et al.* (64), described the preparation of the previously unreported diol, β-dihydroequilin. They reduced the ketosteroid equilin with aluminum isopropoxide to a mixture of α- and β-dihydroequilin, and separated the two isomers on a *Celite:0.4 N sodium hydroxide* column similar to that described above. The β-isomer was eluted with benzene and the α-isomer with ether. Using the same partition system, Banes, *et al.* (65), resolved the "isoequilin A" of Hirschman and Wintersteiner (66) into two major constituents, 8-dehydro-14-isoestrone and 9-dehydro-14-isoestrone. They also isolated the latter compound from equine pregnancy urine extracts.

Beall and Grant (67) showed that the water-soluble conjugated estrogens in concentrates prepared from equine pregnancy urine can be extracted with chloroform from aqueous solution as their cyclohexylamine complexes. However, troublesome emulsions frequently formed during the extraction with conventional liquid-liquid extraction techniques. Carol, *et al.* (68), employed partition chromatography for the extraction. They mixed the sample with Celite 545, added water to dissolve the conjugated estrogens in the preparation and simultaneously constitite an immobile phase, and transferred the mixture to a column. Chloroform eluted the nonconjugated estrogenic materials, together with extractable impurities from the urine concentrate and the soluble tablet excipients. This elution was followed by a chloroform solution of dicyclohexylamine acetate which eluted the chloroform-soluble complex. Graham (69) later found that chloroform eluted significant proportions of conjugated estrogens from tablets containing surface-active agents in their formulations, and substituted ether for chloroform as pre-wash. A mixture of ether and methylene chloride was used to dissolve the dicyclohexylamine acetate and elute the conjugated estrogen complex. In an extension of this procedure, Graham (36) passed the elute containing the conjugated estrogen-dicyclohexylamine complex directly onto a second column, in which concentrated hydrochloric acid is the immobile phase. In a single operation the complex is dissociated, the conjugated estrogen cleaved, and the liberated estrogen eluted.

Snair and Schwinghamer (70) employed partition on *Celite 545 : polyethylene glycol 600* for the assay of estradiol propionate in oil solution. Without the chromatographic purification, sterols present in the oil base interfered with the colorimetric determination of the liberated estradiol. They reported analyses of about 115% for samples in sesame oil and of about 90% for solutions in corn, cottonseed, or peanut oil. After the purification, recoveries in the range of 99.6% to 102.4% were obtained for all samples.

Wolff (71) used a *Celite 545:nitromethane* column, with *n*-heptane as mobile phase, in the quantitative determination of progesterone in oil. Chromatography yielded pure progesterone uncontaminated by oil constituents.

Butt, *et al.* (72), separated progesterone, desoxycorticosterone, and testosterone on a Hyflo Super-Cel column, using 70% methanol as stationary phase and *n*-hexane as mobile phase.

Carol (73) used Celite 545, with 80% ethanol as stationary phase and iso-octane as mobile phase, for the quantitative separation of progesterone and testosterone in commercial aqueous suspensions.

Katzenellenbogen, *et al.* (40), separated androsterone and 5β-androsterone on a silica gel column, using absolute ethanol as immobile phase and petroleum ether–methylene chloride (1:1) as eluant. Bergström and Sjövall (74) employed reverse-phase partition to separate the cholic acids. They used siliconized kieselguhr, prepared as described by Howard and Martin (34), as supporting phase, chloroform-heptane (9 + 1) as immobile phase, and 58% methanol as mobile phase. Cholic acid, deoxycholic acid, and lithocholic acid were eluted in order, with recoveries of 70 to 90%. Hauton (75) applied gradient elution to the same separation. Siliconized kieselguhr was used as support and chloroform–*sec*-octanol (1 + 1) as immobile phase. The mobile phase entered the column from an apparatus consisting of two reservoirs, one containing methanol and the other water, delivering a mixture with a continuously increasing concentration of water. Sharp separations of cholic, deoxycholic, and lithocholic acids were obtained, with recoveries of 97 ± 2%.

Baker, *et al.* (19), isolated steroids from a hog adrenal extract, using Solka-Floc as support, propylene glycol as immobile phase, and toluene as mobile phase. They mixed the extract with Solka-Floc and placed it above the propylene glycol segment of the column. The oily components were eluted initially, followed by cortisone and hydrocortisone. Haines (76) used a *silica gel*:*ethylene glycol* column, with cyclohexane–methylene chloride as mobile phase, to separate (in order) 11-desoxycorticosterone, corticosterone, cortisone, and hydrocortisone. Heftmann and Johnson (77) separated 11-desoxycorticosterone, 11-dehydrocorticosterone, cortisone, hydrocortisone, and Δ-4-pregnene-17α, 21-diol-3,20-dione, on a *silicic acid*:*water* column. They used as developing phase petroleum ether mixed with increasing proportions of methylene chloride, added as described by Donaldson, *et al.* (52). Morris and Williams (78) separated corticosteroids from lipids by reverse-phase chromatography, using petroleum ether as immobile phase on siliconized Celite with 50% methanol as mobile phase. The initial eluate contained a mixture of 11-dehydrocorticosterone, corticosterone and cortisone, and the next fraction contained a mixture of cortisone and hydrocortisone. These were further resolved on a *Celite*:*ethylene glycol* column, with petroleum ether–toluene (2 + 8) as mobile phase.

Banes (79) described a procedure for the complete chemical assay of adrenal cortex extracts in their commercial dosage forms. Two columns were employed for the complete separation of the steroids. In the first column, Celite 545 was the support, formamide-water (1 + 1) the immobile phase, and benzene the mobile phase. The first fraction of eluate contained a mixture of 11-dehydrocorticosterone and corticosterone, the second fraction cortisone, and the final fraction hydrocortisone. The mixture of 11-dehydrocorticosterone and corticosterone was completely resolved upon the second column in which formamide-water (4 + 1) was immobile phase on Celite and benzene-isooctane (3 + 2) the mobile phase. In evaluating the procedure he obtained recoveries of 95 to 102% from a mixture of 1 to 2 mg. of each of the four compounds.

Jones and Stitch (80) separated dehydroepiandrosterone from epiandrosterone on a *silicic acid*:*nitromethane* column, using 3% chloroform in petroleum ether as mobile phase.

Stoll and Kreis (22) applied partition chromatography to the separation of the squill glycosides. They used cotton linters as supporting phase, water as immobile phase, and ethyl acetate as mobile phase. Hegedüs, *et al.* (26), chromatographed the strophanthus glycosides on *Hyflo Super-Cel*:*water*, with mobile solvents of increasing polarity, progressing from petroleum ether-benzene to chloroform. They isolated several new glycosides.

Banes and Carol (81) described a procedure for the quantitative determination of digitoxin, gitoxin, and digitoxigenin in digitoxin tablets. They used a mixture of formamide and water (2 + 1) as immobile phase on Celite 545. An extract of the sample was added to the column and the chromatogram was developed with chloroform-benzene (1 + 9). The forerun contained digitoxigenin, which was followed by digitoxin. To recover gitoxin, they extruded the column contents into a separator and extracted the gitoxin with chloroform. Recoveries of 95.6 to 102.3% of standard preparations were obtained. In a modification of this procedure (43), a solution or suspension of the sample itself in formamide-water was used as immobile phase. Iso-octane was used to elute digitoxigenin, benzene-chloroform (3 + 1) the digitoxin, and chloroform the gitoxin. The gitoxin content of digoxin preparations was determined (82) by converting it to dianhydrogitoxigenin and

isolating it by elution with iso-octane from a *Celite:50% ethanol* column.

Antibiotics.—Among the earliest applications of partition chromatography was the separation of the penicillins. Catch, *et al.* (3), used a solution of an alkali carbonate with either silicic acid or Hyflo Super Cel as support. Ether or amyl acetate was the mobile phase. Fischbach, *et al.* (83), separated penicillin K quantitatively from mixtures with the other penicillins, on a column of *silicic acid:20% phosphate buffer at pH 6.4*, using ether as mobile solvent. The same authors (11) separated penicillins K, dihydro F, F, and G using a similar system. Recoveries of known mixtures were 99% for penicillin K, 82% for penicillin dihydro F, 99% for penicillin F, and 98% for penicillin G. Low recoveries of penicillin dihydro F (80–85% in all cases) was attributed to destruction during the treatment. Higuchi and Peterson (84) identified the individual penicillins in mixtures by hydrolyzing them to obtain their characteristic acids and separating the acids on Celite columns. Either 30 *N* sulfuric acid or a mixture of sulfuric and phosphoric acids (8 + 11) was used as immobile phase and benzene as mobile phase.

Higuchi, *et al.* (85), separated intact chloramphenicol from its degradation products, eluting the latter from a *silica gel:water* column with chloroform and the intact antibiotic with chloroform–ethyl acetate (2–10%).

Analgesics.—Higuchi and Patel (86) applied partition chromatography to the analysis of mixtures of acetylsalicylic acid, acetophenetidin, and caffeine. After removing the acetylsalicylic acid in a separator, they separated the other two components on a column of 20 Gm. of silicic acid and 20 ml. of water. Chloroform–isopropyl ether (1 + 3) was used to elute the acetophenetidin, and chloroform to elute the caffeine. Smith (87) used Celite 545 as support and chloroform-ether (1 + 9) as eluant for the same separation. Banes (43) used a column segment of 5 ml. of 2 *N* hydrochloric acid on 5 Gm. of Celite 545 for the separation. Above this was placed a segment prepared by mixing 6 Gm. of Celite with 6 ml. of a solution of the caffeine and acetophenetidin in formamide-water (2 + 1). Ether was used for elution of the acetophenetidin and chloroform for the caffeine. Levine (59) used a two-stage column to separate the three components without prior extraction of acetylsalicylic acid. The first stage, 2 ml. of 4 *N* sulfuric acid on 2 Gm. of Celite, trapped caffeine from an ether solution, and the second stage, 2 ml. of 1 *N* sodium bicarbonate on 2 Gm. of Celite, trapped acetylsalicylic acid as its salt. Ether eluted the neutral acetophenetidin and chloroform the caffeine. To recover the acetylsalicylic acid, the bicarbonate stage was acidified *in situ* with a solution of acetic acid in chloroform, and the liberated acetylsalicylic acid was eluted with chloroform. The caffeine and acetylsalicylic acid eluates were received directly in volumetric flasks and their concentrations measured spectrophotometrically, without further treatment. Indemans and Mulder (88) substituted 4 *N* sodium hydroxide for the 1 *N* sodium bicarbonate to increase the capacity of the column, in order to chromatograph samples large enough to permit gravimetric measurement. Stroes (89) reported that the 50 ml. of chloroform specified by Levine is insufficient to elute the caffeine quantitatively, and that 100 ml. is necessary. In a collaborative study of the procedure conducted by Smith (90), however, quantitative recoveries with the specified volumes of eluants were obtained.

Heuermann and Levine (58) separated combinations of acetylsalicylic acid, acetophenetidin, and caffeine with codeine, antihistamines, and/or barbiturates, using multiple columns. For the combinations with codeine or the antihistamines, the sodium bicarbonate and sulfuric acid stages were placed in separate columns arranged in such a manner that effluent from one ran directly onto the second. After elution of the acetylsalicylic acid, acetophenetidin, and caffeine exactly as in the procedure for this combination alone, the codeine or antihistamine was eluted after conversion from its salt to the chloroform-soluble free base with a solution of triethylamine in chloroform. For combinations with barbiturates, a third column, with tripotassium phosphate solution as immobile phase, was placed in series between the sodium bicarbonate and the sulfuric acid columns. The very weakly acidic barbiturate passed through the sodium bicarbonate column, but was held on the more strongly alkaline tripotassium phosphate column, while the other components passed through. The barbiturate was eluted after acidification of the column with acetic acid in chloroform.

To separate acetophenetidin from isopropylantipyrine, Indemans and Mulder (88) suspended the mixture in 4 *N* sulfuric acid, precipitated the isopropylantipyrine with potassium ferrocyanide, added Celite, and transferred the mixture to a column. Ether eluted the acetophenetidin alone.

The separation of caffeine from acetophenetidin by the procedure of Higuchi and Patel (86) is based upon partition of caffeine between water, the immobile phase, and the mobile phase. It is for this reason that the relatively long column, containing 20 ml. of immobile phase, is required. In the procedure of Levine (59), on the other hand, the separation is based upon salt formation. Caffeine, while feebly basic, will form salts with strong acids; therefore only 2 ml. of immobile phase is sufficient to trap the caffeine as its salt from ether solutions. If a less concentrated acid (*e.g.*, 2 *N* sulfuric acid) is used, the caffeine is not quantitatively retained; if a more concentrated acid (*e.g.*, 6 *N* sulfuric acid) is used, the caffeine is not eluted quantitatively by the specified volume of chloroform.

The procedure for the analysis of acetylsalicylic acid combinations was adapted to apply to the analysis of acetylsalicylic acid in its various dosage forms, including flavored, colored, buffered, and enteric-coated tablets (37). The same publication describes the determination of the free salicylic acid content of acetylsalicylic acid. Ferric chloride solution is used as immobile phase to retain salicylic acid as its complex while acetylsalicylic acid, which has no phenolic hydroxyl group, passes through. In this separation, only dilute solutions of ferric chloride will remove salicylic acid from chloroform, and acetylsalicylic acid removes ferric chloride from the column as the chloroform-soluble salt. A balance must therefore be maintained to provide a sufficiently low concentration of ferric chloride to trap the salicylic acid, and at the same time provide a sufficiently large quantity to insure an excess over that removed by the acetylsalicylic acid. For a 200-mg. sample, an effective balance was achieved by using 5 Gm. of Celite and 7 ml. of 2% ferric chloride solution. The salicylic acid remains on the column as its purple ferric ion complex. The salicylic acid is eluted after the complex is dissociated with a solution of acetic acid in chloroform.

Barbiturates.—In a procedure for the determination of phenobarbital in mixtures with salicylates (91), a chloroform solution of the mixture is passed over a *Celite 545:2 M dipotassium phosphate* column. The salicylates remain on the column while phenobarbital passes through. Byers (92) applied this procedure to phenobarbital–acetylsalicylic acid combinations. Lach, *et al.* (93), used a column of pH 9.2 buffer on Celite 535 to separate diphenylhydantoin from phenobarbital. Chloroform eluted the diphenylhydantoin and butanol-chloroform (1 + 9) the phenobarbital. Recoveries of 99 to 100% were obtained. Sabatino (94) separated phenobarbital from other barbiturates (secobarbital, pentobarbital, amobarbital, or butabarbital) on a Celite column, using formamide-water (2 + 1) as immobile phase. Iso-octane–chloroform (1 + 1) eluted the barbiturate other than phenobarbital, and chloroform eluted the latter. Stroes (89) separated phenobarbital from aprobarbital on a Celite column with pH 9 buffer as immobile phase. Chloroform eluted the aprobarbital and ether the phenobarbital. The separation of barbiturates from combinations with acetylsalicylic acid, acetophenetidin, and caffeine (58) has been described above.

Alkaloids.—Graf (32) applied a combination of adsorption and partition chromatography to the assay of alkaloid-bearing crude drugs. Although adsorption has a much greater role in this procedure than in the others discussed in this review, sufficient features of partition chromatography are retained to warrant its inclusion. In this procedure, an alkaline aqueous suspension of the powdered crude drug, constituting the immobile phase, is stirred with sufficient activated alumina to prepare a dry mixture. The alkaloid is eluted with an immiscible solvent. Strong or weakly polar solvents may be used, depending upon the activity of the alumina. This procedure was used in the assay of lobelia, ephedra, nux vomica, hydrastis, cinchona, yohimbe, ipecacuanha, solanacea, and other drugs. In applying this procedure to opium, Graf found alumina to be too strongly adsorbent and substituted kieselguhr as supporting phase. Ethylene chloride was used to elute the alkaloids other than morphine, along with the coloring matter. The morphine was eluted with chloroform-isopropanol (3 + 1). When alumina was used as support, elution of the by-alkaloids and coloring matter with ethylene chloride was incomplete; these contaminated the morphine in the ensuing elution.

Belladonna Alkaloids.—Schill and Ågren (42) extracted belladonna alkaloids in a similar manner. They made the crude extract alkaline with sodium carbonate, sorbed the solution on kieselguhr, and packed the mixture in a column. They passed the chloroform eluate from this column, containing the alkaloids together with neutral extractives, over a second kieselguhr column in which 0.5 *M* phosphoric acid was immobile phase. This column retained the alkaloids, while the neutral components passed through. They eluted the alkaloids with

ammonia-saturated chloroform, which made the immobile phase alkaline *in situ*.

Atropine, in solutions containing phenol as preservative, has been separated from its hydrolytic product, tropic acid (95). Two columns were used in series. The upper contained the sample solution, made alkaline with sodium bicarbonate, as immobile phase on Celite 545; the lower column contained *Celite:0.2 N sulfuric acid*. Chloroform extracted the atropine and phenol from the upper column; the alkaloid was retained on the lower column, while phenol passed through. Tropic acid was eluted from the upper column with a solution of acetic acid in ether. To recover the atropine from the lower column, a segment of 1 Gm. of Celite and 1 ml. of concentrated ammonium hydroxide was added to the column, and chloroform was passed over it. The added segment was effective in saturating the chloroform with ammonia.

The combined effect of partition coefficient and dissociation constant upon the separation of alkaloids has been discussed above. Evans and Partridge (50) applied this effect to the separation of hyoscine and hyoscyamine. They used kieselguhr as support and, as immobile phase, a series of phosphate buffers ranging in pH from 6.2 to 7.4. The 6.2 buffer was most efficient in separating the two alkaloids. At this pH, the hyoscine is completely eluted with ether long before any hyoscyamine appears in the eluate. The latter is readily eluted with chloroform. The same authors (96) used pH 7.3 buffer to separate the alkaloids of Australian *Datura Ferox* and Indian henbane. Using ether as mobile phase, they eluted (in order) hyoscine, hyoscyamine, and metaloidine; chloroform then eluted tropine from extracts of the latter species. Evans and co-workers (97–100) isolated a number of alkaloids from the individual plant parts of several *Datura* species on *kieselguhr:phosphate buffer* columns at pH 5.4 to 6.6, using petroleum ether, ether, and chloroform as developing solvent. Santoro and Matz (101) separated the belladonna alkaloids from dosage forms containing chlorpheniramine and phenylpropanolamine, using a Hyflo Super-Cel column with pH 7.0 buffer as immobile phase. Chlorpheniramine was eluted with carbon tetrachloride–cyclohexane (1 + 1) and the belladonna alkaloids were eluted with chloroform. Phenylpropanolamine remained on the column.

Veratraea Alkaloids.—In a series of publications, Parks and associates described the application of partition chromatography to the separation of the sabadilla alkaloids. Hennig, Higuchi, and Parks (102) using *silicic acid:pH 8.5 phosphate buffer*, with chloroform containing 1–3% ethanol as mobile phase, separated the water-soluble sabadilla alkaloid group into five fractions, including the new alkaloid sabatine. Svoboda and Parks (103) prepared pure veratridine and cevadine by chromatography of a crude alkaloid mixture on *silicic acid:pH 4.0 or 4.25 buffer*. Stuart and Parks (104) chromatographed fractions obtained by countercurrent distribution on a *silicic acid:pH 5.0 buffer* column, with chloroform as eluant. They isolated the pure alkaloid vanilloylcevine. Mitchner and Parks (29) used two systems, silicic acid as support and pH 5.90 buffer with benzene as mobile phase, or pH 3.95 buffer with chloroform as mobile phase, in an investigation of the separation of veratridine and cevadine. They compared the chromatographic separations with those obtained by countercurrent distribution, and found that effects other than partition occurred on the column. They attributed this to the tailing effect due to adsorption.

Brown and Kupchan (24) incorporated bromthymol blue in the immobile phase of a Celite column to monitor the separation of the alkaloids, the location of the bands being shown by the indicator. The polar layer of an equilibrated mixture of ethylene chloride–Skellysolve B, methanol, and water (3 + 12 + 2 + 0.24) was used as immobile phase and the nonpolar layer as developing phase. They separated commercial "Veratrine" into two completely homogeneous bands, cevadine and veratridine. Using the same system, they obtained three bands from *Zygadenus paniculatus:* the first contained neogermitrine, the second a mixture of neogermidine and germidine, and the third pure zygacine.

Levine and Fischbach (30) separated "protoveratrine" into its components, protoveratrines A and B, on a Celite column, using as immobile phase pH 3.5 buffer–ethylene glycol (2 + 1). Benzene eluted the protoveratrine A and ethylene chloride the protoveratrine B. When aqueous buffer was used without the glycol, losses occurred due to adsorption. The same authors (105) modified the procedure for application to commercial protoveratrine preparations which contained associated alkaloids. They substituted pH 3.0 buffer–ethylene glycol (2 + 1) as immobile phase. Carbon tetrachloride–ethylene chloride (3 + 2) eluted protoveratrine A; ethylene chloride eluted alkaloids intermediate between protoveratrines A and B in hydrophilic strength; chloroform–ethylene chloride (2 + 1) eluted protoveratrine B; and finally chloroform

eluted the more strongly hydrophilic alkaloids. Paper chromatography was used to monitor the separations.

Hegi and Flück (106) used *kieselguhr:pH 4.2 buffer* columns for the separation of pairs of veratrum alkaloids. They used chloroform-ether (40 + 160), chloroform-ether (90 + 110), and chloroform to separate the alkaloid pairs protoveratrine A–protoveratrine B, germitetrine, and jervine-veratrobasine. They used this same chromatographic procedure in isolating new alkaloids from the above-ground portions of *Veratrum album* (107). Kupchan and Gruenfeld (108) resolved the components of a commercial *Veratrum viride* alkaloid preparation on a *Celite:pH 4.25 buffer* column, eluting successively with benzene, benzene-chloroform, chloroform, and chloroform-methanol. They rechromatographed certain of the fractions on alumina, and isolated and identified a total of 13 alkaloids.

Ergot Alkaloids.—Brindle, *et al.* (109), separated the water-insoluble ergot alkaloids ergocristine and ergocristinine. They used Solka-Floc as support, pH 3.4 buffer as immobile phase, and ether as mobile phase. Carless (57) used a column of pH 3.0 buffer on ashless cellulose powder in the chromatography of the ergot alkaloids. With ether as eluant, the alkaloids moved down the column in the order ergocristinine, ergocristine, ergosine, and ergotamine. The water-soluble alkaloids, ergonovinine and ergonovine, remained at the top of the column. Ergosine and ergotamine moved very slowly, requiring inordinate volumes of eluate. Increasing the pH of the immobile phase accelerated their movement, but resulted in incomplete separation of the faster-moving ergocristinine and ergocristine. Pyridine (0.1%) was added to the developing solvent, thereby establishing a pH gradient on the column; the pH was raised from 3.0 to 4.8 after passage of 100 ml. of solvent. With this procedure, the slower moving alkaloids were eluted more rapidly and the zones compacted. To elute the water-soluble alkaloids, the column was made alkaline with a solution of diethylamine in ether. Recovery studies using the individual pure alkaloids yielded 90–95% except for ergotamine, for which recoveries were about 80%.

Graf and Neuhoff (110) described the quantitative determination of the water-insoluble and water-soluble groups of ergot alkaloids. They triturated the sample of ground ergot with ammonium hydroxide and methanol, added sufficient silica gel to prepare a dry mixture, and transferred it to a column above a segment of *silica gel:pH 7 buffer*. They eluted the alkaloids with chloroform-trichloroethylene (1 + 1), monitoring the separation by fluorescence under ultraviolet illumination. In control experiments with mixtures of ergotamine and ergonovine they obtained recoveries of 96 to 100%. Their assays of crude ergot gave somewhat higher values than those obtained by non-chromatographic procedures, particularly for the water-soluble group.

Alexander and Banes (111) separated the water-soluble and water-insoluble groups on a *Celite:0.1 M citric acid* column. They passed a chloroform solution of ergot extract over the column and eluted the water-insoluble group with chloroform. They then extruded the column, added sodium bicarbonate to render the immobile phase alkaline, and reconstituted the column. The water-soluble group of alkaloids was eluted with chloroform. Recoveries of 95 to 98% of ergonovine were obtained. To determine the extent to which the water-insoluble alkaloids appear in the water-soluble alkaloid fraction, they chromatographed the pure water-insoluble alkaloids ergotamine and ergocristine by the same procedure. The quantity of the alkaloids found in what would be the water-soluble fraction was equivalent to no more than 3% of the ergonovine present in a normal sample. These authors, like Graf and Neuhoff (110), found that the chromatographic procedure gave higher values, particularly for the water-soluble group, than the conventional shake-out procedure of the National Formulary XI (112).

Van de Langerijt (113) separated ergotamine and ergotaminine on a *Celite:formamide* column, using benzene-petroleum ether (9 + 1) as developing solvent. Ergotaminine was eluted first, followed by ergotamine. The cutting of fractions was monitored by fluorescence of bands under ultraviolet light. Lysergic acid and lumiergotamine present in the sample remained on the top of the column.

Alexander (114) described a procedure for the determination of ergotamine and ergotaminine in its dosage forms, including combinations with acetophenetidin, caffeine, barbiturates, etc. The sample was first chromatographed on a column of *Celite:50% citric acid solution* with ether. The ergot alkaloids remained on the column while the neutral or acidic components passed through. The column was extruded, the aqueous phase was made alkaline, and the combined ergotamine and ergotaminine

were extracted with chloroform. The extract was passed over a second Celite column, in which 25% (w/v) citric acid was the immobile phase. Ergotaminine was eluted with chloroform, with recoveries of 98 to 100%. The column was extruded and ergotamine extracted as before; recoveries of 97 to 98% were obtained. Pin Liang and Tonh-Hui Chou (115) separated ergonovine from the water-insoluble ergot alkaloids on a *Celite:pH 3.4 buffer* column. Chloroform eluted the latter group; ergonovine remained on the top of the column, and was eluted with ammonia-saturated chloroform. Recoveries of over 95% were obtained. Voight and Kaehler (116) adapted a paper chromatographic separation of ergot alkaloids to a column, using cellulose powder as support, 0.2% tartaric acid as immobile phase, and ether-acetone-water (2:4:2) as developing solvent. The peptide alkaloids were eluted first, followed by the clavine fraction. Ammonium hydroxide (1 drop per 100 ml.) was then added to the eluant. Ergonovine, ergonovinine, and lysergic and isolysergic amides were eluted serially.

Rauwolfia Alkaloids.—Pure reserpine was isolated from commercial reserpine preparations by chromatography on a Celite column, using a mixture of 0.05 *M* citric acid and alcohol (5 + 2) as immobile phase and chloroform–alcohol–iso-octane (100 + 40 + 200) equilibrated with water as eluant (117). After a forerun was discarded, spectrophotometrically pure reserpine was eluted.

The weakly basic alkaloids, mainly reserpine and rescinnamine, were separated from *Rauwolfia serpentina* preparations, with a slightly modified immobile phase (118). Chloroform was used as eluant, and 0.5 *N* sulfuric acid and 10% dipotassium phosphate were used as immobile phase on separate segments of the column, to separate reserpine from its acidic degradation products and the more polar alkaloids (119).

Hayden, *et al.* (120), separated the three weakly basic rauwolfia alkaloids, reserpine, deserpidine, and rescinnamine. They equilibrated *n*-heptane, chloroform, morpholine, and formamide (715 + 110 + 1 + 25), and used the lower layer as immobile phase and the upper layer as eluant. With a column of 25 Gm. of Celite and 20 ml. of immobile phase, and quantities of about 1.5 mg. of alkaloid, they found that after a 125 ml. forerun, deserpidine was eluted in the next 75 ml.; after the next 25 ml., reserpine was eluted in 110 ml.; and after the following 60 ml., rescinnamine was eluted in the next 200 ml. Recoveries of deserpidine, reserpine, and rescinnamine averaged 99%, 97%, and 95%, respectively.

Opium Alkaloids.—Lindblad and Ågren (121) used a *Hyflo Super-Cel:pH 2.0 phosphate buffer* column to separate several opium alkaloids. Ether eluted narcotine, chloroform eluted papaverine, and ammonia-saturated chloroform eluted codeine. Büchi and Huber (122), using a *kieselguhr:pH 4.6 buffer* column and benzene-ether (1 + 3) as developing solvent, eluted first a mixture of narcotine and papaverine and then thebaine. Codeine was eluted with ammonia-saturated chloroform. Lach, *et al.* (123), separated morphine from pseudomorphine on a *Celite 535:pH 6.5 buffer* column. Chloroform containing 15% butanol eluted pure morphine, leaving the pseudomorphine on the column. Yeh and Lach (124), using a similar column, eluted degradation products of pseudomorphine with chloroform containing 5% butanol; pure morphine was then recovered as before.

Other Alkaloids.—Welsh (125) determined epinephrine and norepinephrine in mixtures by chromatographing their triacetyl derivatives on a *Celite:water* column. Benzene eluted the triacetyl epinephrine and chloroform the triacetyl norepinephrine. Buffer (pH 4) was used as immobile phase and chloroform as eluant to separate triacetylepinephrine from its degradation products (126).

Jensen and Svendsen (53) separated strychnine and brucine on a kieselguhr column with pH 7 buffer as immobile phase. The strychnine was eluted with ether and the brucine with chloroform.

Chilton and Partridge (28) separated the pomegranate alkaloids of the commercial mixture pelletierine tannate, using powdered glass as support and pH 6.8 buffer as immobile phase. They found that with kieselguhr excessive loss was incurred due to adsorption. The aconite alkaloids were separated on a *kieselguhr:pH 7 buffer* column, with first ether and then chloroform as mobile phase (127).

To avoid gelation which occurs in the separator on extraction of pilocarpine from solutions containing methylcellulose, the sample solution has been used to constitute the immobile phase on Celite (128). Chloroform eluted the alkaloid quantitatively.

Salts of Bases.—The classical analytical procedures for alkaloids are based upon the general rule that the nondissociated form (free base) will enter the organic phase and the dissociated form (the salt) will enter the aqueous

acidic phase during distribution between the immiscible solvents. With selection of the proper acid, however, it is possible to extract the specific salt of most alkaloids and organic bases with chloroform directly from the acidic aqueous solution. Although the distribution coefficient may be relatively unfavorable, quantitative recoveries are obtained because of the efficient extraction which is obtained by column partition chromatography. Application of this means of distribution to the analysis of a large variety of alkaloids and related bases in their dosage forms is thus feasible. Schill and Ågren (129) applied this property to the separation of hyoscyamine hydrochloride from scopolamine hydrochloride. They used a *kieselguhr:1 N hydrochloric acid* column for the separation; chloroform eluted the hyoscyamine hydrochloride quantitatively while the scopolamine salt remained on the column. The authors attributed the separation simply to the solubility of hyoscyamine hydrochloride and the insolubility of scopolamine hydrochloride in chloroform. It has been shown (31) that solubility of the salt in chloroform is only one factor governing the extractability of the salt from aqueous solution. Salts having comparable solubility in chloroform may vary widely in their partition characteristics. In the distribution of diphenhydramine hydrochloride and of cyclizine hydrochloride (both of which are readily soluble in chloroform) between chloroform and aqueous hydrochloric acid solution, the proportion of diphenhydramine in the chloroform phase at equilibrium increases with increasing concentration of acid, while that of cyclizine decreases.

The procedure of Haddock and Evers (130) for the separation of strychnine from quinine was adapted to partition chromatography (43). The separation of the two alkaloids was more complete, the analyses more accurate, and the manipulations much more simple than in the original procedure, in which the extractions were performed in separators. A *Celite 545:2 N hydrochloric acid* column was used for the separation; chloroform eluted strychnine while quinine was retained on the column. Dihydrocodeinone was separated from pheniramine on a similar column; the latter is retained on the column while dihydrocodeinone passes through (131).

It has been shown that acids other than hydrochloric may be used to effect the differential extraction of salts of alkaloids and organic bases, and that by proper selection of acid, significant degrees of specificity can be achieved. Nitric acid (1 *N*) was used as immobile phase (132) for the separation of codeine from pyrilamine, methapyrilene, and pheniramine; chloroform eluted codeine from this column while the antihistamines were retained. The chloroform eluate was passed onto a *Celite:0.5 N sulfuric acid* column, which retained the codeine while the neutral and acidic extractives of the sample (*e.g.*, a cough syrup) passed through. This partition system was not applicable to the separation of codeine from phenindamine, since this antihistamine is not retained on the nitric acid column. To separate this combination, a *Celite:0.5 N sulfamic acid* column was used, with chloroform as mobile phase. The phenindamine passed through while codeine was retained.

Miller (133) reported that while the chromatographic procedure of Banes (43) for the determination of strychnine in elixir of iron, quinine, and strychnine was applicable to freshly prepared elixir, high values were obtained for aged samples. He showed (134) that this was caused by the partial degradation of quinine to one or more products not separated from strychnine in the chromatographic step. Pure strychnine was isolated from the aged elixir on a three-stage column (135). In the first stage, a solution of *p*-toluenesulfonic acid in the sample constituted the immobile phase. The second stage was *Celite:2 N hydrochloric acid*, and the third stage *Celite:1 N tartaric acid*. Ether, passed over the first stage alone, eluted one or more of the quinine degradation products which accompanied strychnine in the previous procedure. Chloroform then eluted the quinine, strychnine, and the remaining quinine degradation products. The chloroform eluate was passed through the second stage, which retained the quinine, and over the third stage, which retained the strychnine, while the remaining quinine degradation products passed through. Strychnine was recovered from this stage with chloroform-triethylamine as described above. If 1 *N* sulfuric acid was used rather than tartaric acid in the third stage, the final separation was not obtained; the quinine degradation product was retained together with the strychnine.

Levine and Ottes (136) showed that the analysis of alkaloidal preparations by extraction of the salt with chloroform from aqueous solution of *p*-toluenesulfonic acid is applicable to a large variety of alkaloids. A solution of the dosage form in 10% *p*-toluenesulfonic acid is used as immobile phase on Celite. Ether elutes neutral and acidic constituents of the sample, and chloro-

form elutes the alkaloid-*p*-toluenesulfonic acid complex. This eluate is passed over a *Celite:1 N sodium hydroxide* column, which retains the acid moiety while the alkaloid passes through. Centigram quantities of alkaloid can be isolated by this procedure. Quantitative recoveries were obtained with strychnine, quinine, atropine, codeine, emetine, physostigmine, dihydrocodeinone, quinidine, and homatropine. Low recoveries resulted with cocaine, apomorphine, and arecoline, while morphine, pilocarpine, procaine, and phenylephrine were retained completely. This procedure was adapted (31) to the quantitative analysis of those antihistamine preparations for which the procedure *Salts of Organic Nitrogenous Bases* is official in U.S.P. XVI (137). *p*-Toluenesulfonic acid could not be used for all of the compounds, since the ether used in the purification step eluted varying quantities of several bases. It has been found that this phenomenon is the cause of the low recoveries of cocaine, arecoline, and apomorphine mentioned above. For these compounds the *p*-toluenesulfonic acid was replaced by 2 *N* hydrochloric acid or, in the case of those which were eluted by ether from this acid, 5% sulfamic acid solution. In the final procedure as adopted, 10% *p*-toluenesulfonic acid is used for chlorpheniramine, doxylamine, pyrilamine, and tripelennamine; 2 *N* hydrochloric acid for chlorcyclizine, cyclizine, and diphenhydramine; and 5% sulfamic acid for phenindamine and promethazine. Tailing occurred during the elution of several of the compounds with chloroform, but was completely eliminated by adding 1% glacial acetic acid to the chloroform eluant; quantitative recoveries of all of the compounds were obtained.

Miscellaneous Applications.—Theivagt and Campbell (138) separated vitamin D from vitamin A on a Celite 545 column, using polyethylene glycol 600 as immobile phase and isooctane as eluant. The *R* values of the two vitamins, calculated by the formula developed by Martin and Synge (6), predicted that vitamin D should come off the column in $^1/_3$ the volume required to elute vitamin A. The experimental values were very close to the theoretical. The procedure was applied to pharmaceutical products, including multivitamin tablets and liquids and fish liver oils. Quantitation of the isolated vitamin D agreed closely with results obtained by the U.S.P. bioassay. A similar chromatographic system, substituting *n*-hexane for iso-octane as eluant, was used (139) to separate vitamin A from β-carotene, vitamin E, and oxidized vitamin A. The progress of the vitamin A band in the column was monitored by occasional examination in low intensity ultraviolet light. Recoveries of 98 to 101% were obtained for vitamin A added to multivitamin preparations; 98 to 103% from mixtures with oxidized vitamin A; 95% for mixtures with vitamin E; and 92 to 102% for fortified margarine.

Higuchi, *et al.* (140), separated the parabens (*p*-hydroxybenzoic esters) on a silicic acid column, using methanol–water–sulfuric acid (7.5 ml. + 3 ml. + 1 drop) as immobile phase and carbon tetrachloride–Skellysolve B (1 + 1) as eluant. The parabens were eluted in the order butyl, propyl, ethyl, methyl.

In a procedure for the determination of piperazine by acetylation and extraction of the diamide, Perlmutter (141) reported the usual separator extraction to be quite laborious. Because of the unfavorable distribution coefficient, a minimum of ten extractions is necessary for quantitative recovery. To improve the procedure, he mixed an alkaline solution of piperazine with Celite 545 and stirred the mixture with acetic anhydride to form the diamide. The material was transferred to a column (142) and the diamide was eluted with 200 ml. of chloroform.

In the determination of glyceryl trinitrate in its dosage forms, Levine and Hohmann (143) separated the ester from its degradation products chromatographically. They eluted the ester with iso-octane from a column in which a solution of the sample in 50% acetic acid was the immobile phase and Celite 545 the support.

De Ropp (144) chromatographed the phenolic fraction of *Cannabis sativa* "red oil" on a *Celite:dimethylformamide* column with cyclohexane. He separated tetrahydrocannabinol, cannabinol, and cannabidiol in that order.

Schwartz, *et al.* (145), used two chromatographic systems for the determination of naphazoline and its hydrolytic products, 1-naphthylacetic acid and its ethylenediamine monoamide. Chloroform eluted 1-naphthylacetic acid from a *silicic acid:water* column while the amide and intact naphazoline remained on the column. The latter two compounds were separated on a *Celite:pH 8.5 buffer* column. The intact material was eluted with *n*-heptane–chloroform (65 + 35) and the amide with chloroform. The naphthylacetic acid was retained on this column. The chromatographic behavior of the other hydrolytic product, ethylenediamine, was not determined.

Taraszka and Marcus (146) separated adiphenine from its hydrolytic product, β-diethylaminoethanol, on a *silicic acid:pH 4.8 buffer* column, using chloroform as eluant. The adiphenine was eluted and determined by nonaqueous titration. Recoveries of 99.1 to 100.7% were obtained. It was not established whether the second hydrolytic product, diphenylacetic acid, remained on the column, but this compound did not interfere in the titration.

Several procedures have been described in which operations other than simple extraction take place in the partition system. The hydrolysis of the conjugated estrogens on a chromatographic column, in which the hydrolytic reagent, concentrated hydrochloric acid, is the immobile phase, has been described above (22). Levine and Fischbach (30) adapted to the partition column the familiar acid-dye procedure for the determination of alkaloids. They used a solution of chlorophenol red in a mixture of ethylene glycol and pH 3.5 buffer as immobile phase. Portions of chloroform containing various quantities of protoveratrine were passed over the column successively. The eluate contained quantities of dye proportional to the alkaloid content of each aliquot of chloroform. Clark (147) determined phenylephrine in combinations with other bases in formulations such as cough syrups. The sample, made alkaline with dipotassium phosphate, constituted the immobile phase. Chloroform eluted the accompanying bases, such as codeine and antihistamines, together with neutral extractives. A chloroform solution of acetic anhydride was then passed onto the column, acetylating the phenylephrine *in situ*. The product, diacetylphenylephrine, was then eluted quantitatively with chloroform.

APPLICATIONS

Partition chromatography constitutes an integral step in the analysis of four products which are included in the official compendia of the United States: digitoxin, digitoxin injection, and digitoxin tablets (148), based upon the procedure of Banes, *et al.* (81); ouabain and ouabain injection (149), based upon the procedure of Banes and Houk (150); ergotamine tartrate injection (151), based upon the procedure of Alexander (114); acetylsalicylic acid, acetophenetidin, and caffeine capsules and tablets (152), based upon the procedure of Levine (59).

Analytical procedures employing partition chromatography have been adopted as official methods of analysis of the Association of Official Agricultural Chemists, following validation by collaborative studies. These include: ergotamine in tablets, applicable in presence of caffeine, acetophenetidin, phenobarbital, and belladonna alkaloids (153), based upon the procedure of Alexander (154); elixir of iron, quinine, and strychnine (155, 156), based upon the procedures of Banes (43), Miller (134), and Levine (135); phenobarbital in mixtures with salicylates (157), based upon the procedure of Banes (91); phenobarbital in mixtures with acetylsalicylic acid (47), based upon the procedure of Byers (92); acetophenetidin, acetylsalicylic acid, and caffeine (48), based upon the procedure of Levine (59) and collaborative study by Smith (90); β-estradiol (158), based upon the procedure of Haenni, *et al.* (45); norepinephrine in preparations of epinephrine (159), based upon the procedure of Welsh (160); phenylpropanolamine (161), based upon the procedure of Smith (162); digitoxin (163), based upon the procedure of Banes (164); determination of codeine in presence of antihistamines (165), based upon the procedure of Levine (132); determination of conjugated estrogens (166), based upon the procedure of Carol, *et al.* (68), and collaborative study of Banes (167); determination of piperazine (168), based upon the procedure of Perlmutter (142); determination of dihydrocodeinone in presence of antihistamines (169), based upon the procedure of Levine (131).

SUMMARY

The importance of liquid-liquid extraction in pharmaceutical analysis makes column partition chromatography particularly useful in this field. This review of the literature shows how several of the unsatisfactory features of early applications of the technique have been overcome or circumvented. These include availability of material suitable for use as supporting phase, means for limiting or preventing loss due to adsorption, further means for monitoring separations, and simplification of experimental technique. The recent introduction of such variations as multistage columns and the separation of combinations of organic bases by differential solvent extraction from solution in a specific acid facilitates the analysis of complex mixtures which are resistant to analysis by conventional procedures. Communications from colleagues indicate that these developments have given new impetus to the use of column partition chromatography in pharmaceutical analysis.

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Research Articles

Powdered Particle Interactions: Suspension Flocculation and Caking II

By BERNARD ECANOW and ROBERT G. WILSON

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The sulfamerazine suspension system was previously discussed in the literature (2, 3).

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The sulfamerazine suspension system was previously discussed in the literature (2, 3).

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It had been suggested that the flocculation was due to the interaction of the van der Waals forces operating after the zeta potential of the particles had been neutralized by the flocculating agent. This proposal was questioned on two counts (1): (*a*) The particles were in macro-particulate suspension, rather than in colloidal dispersion. Van der Waals forces, although presumably present, have not been shown to be of significance in the type of system studied, where particles are above the colloidal size range. (*b*) The flocculation was virtually immediate, on mixing, and thus was more typical of a chemical reaction than of the reaction rate associated with the action of van der Waals forces. Burton (4) has shown that the coagulation of a colloidal system, following the neutralization of particle charge, is a slow process which depends upon the Brownian movement of the particles.

A bismuth subnitrate suspension which could be flocculated by the addition of monobasic potassium phosphate solution was also reported in the literature (2, 3). The flocculation reaction was discussed in terms of zeta potential and van der Waals forces. This again raised the above questions relative to particle size and speed of the flocculation reaction. The bismuth subnitrate-monobasic potassium phosphate suspension system has in this report been considered from a different viewpoint, with the thought that both flocculation and caking phenomena might be explained on some basis other than that previously proposed.

In the exploratory work, a 2% bismuth subnitrate suspension was reacted with a flocculating agent, and the degree of flocculation was judged empirically from the settling rate. This could be done, since flocculated particles settle more rapidly than do the same particles in a deflocculated state. The words, "flocculated" and "deflocculated," are here used in accordance with the definition given by Fischer and Gans in their chapter in Alexander (5). The following compounds, at a concentration of 1 mmole of phosphate per 100 ml. of suspension, were tested for their flocculating ability.

Flocculating Agent	Relative pH of the Additive in Solution	Flocculating Action
Tri-sodium phosphate	12.0	little
Dibasic sodium phosphate	8.7	little
Monobasic sodium phosphate	4.3	marked
Phosphoric acid	2.2	marked

As can be seen, there was little flocculating action for additive solutions of high pH, but a marked degree of flocculation at lower levels of pH. Since the same concentration of phosphate was present in each case, it seemed that flocculation was pH-dependent. This finding lent credence to the thought that a chemical reaction might be involved.

Bismuth subnitrate for pharmaceutical use is a white, slightly hygroscopic powder. Under the microscope, the powder can be resolved to transparent, orthorhombic crystals averaging 10 to 15 μ in size. The compound may be produced by several quite comparable processes. Perhaps the simplest is to prepare a solution of bismuth nitrate in nitric acid solution of the proper strength and to then pour this solution into a large quantity of water. The bismuth nitrate promptly hydrolyzes, precipitating bismuth subnitrate from an acid medium. Nitric acid is a product of the hydrolysis reaction as well as being present in the initial bismuth nitrate solution. The precipitated bismuth subnitrate is separated by filtration or centrifugation, is carefully dried, and is sold in this form. Because of the conditions of the process, bismuth subnitrate resuspends in water to give a slightly acid medium. An alternate production method (6) is to add sodium carbonate to bismuth nitrate in acid solution until the pH rises to 5.0. At this point, the precipitated bismuth subnitrate is separated and carefully dried as before. Both of the methods discussed will give bismuth subnitrates of variable composition, and the pH obtained on resuspending the solid will be influenced both by the manufacturing process and by the extent to which the material was dried.

Hückel (7) has discussed the "iso-poly bases" at some length. These are compounds which are known to form a variety of weakly basic hydroxides. The hydrolysis of an iso-poly base leads to a hydrated oxide of the compound, or to an oxide complexed with an indeterminate number of water molecules. The composition and structure of the hydroxide, as well as its initial formation, are all strongly pH dependent. Hückel has also said that the size of the iso-poly cations formed can be strongly influenced by the types of anions present in the precipitation medium. The attainment of the hydrolysis equilibrium is described as a slow process, and one in which the initial reversible equilibrium reaction is frequently followed by an irreversible aging process. Because various products may be formed at the different stages of the reaction, and because these products may then be taken to different stages of the aging process, it follows that the end result of the hydrolysis reaction is a product which is variable and complex in its composition.

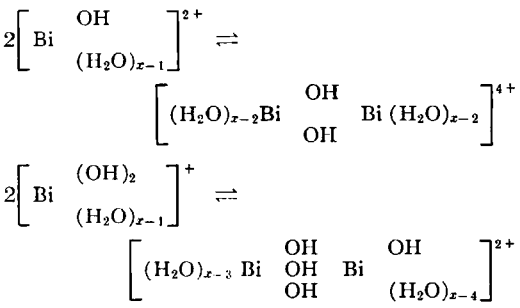
Hückel did not specifically deal with bismuth subnitrate. However, a footnote in his work indicates that the hydrolysis reactions of the bismuth salts are thought to be comparable to those of trivalent chromium. Utilizing Hückel's presentation, but substituting trivalent bismuth for chromium, the following outlines some of the typical reactions of an iso-poly base.

(*a*) The formation of "aquo-cations."[1]

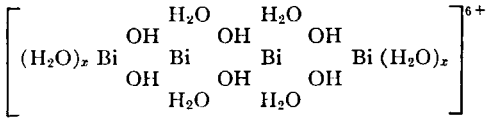
$$Bi^{3+} + xH_2O \rightleftharpoons Bi(H_2O)_x^{3+} \rightleftharpoons \left[\begin{matrix} & OH & \\ Bi & & \\ & (H_2O)_{x-1} & \end{matrix}\right]^{2+} \rightleftharpoons \left[\begin{matrix} & (OH)_2 & \\ Bi & & \\ & (H_2O)_{x-2} & \end{matrix}\right]^{+} \rightleftharpoons \left[\begin{matrix} & (OH)_3 & \\ Bi & & \\ & (H_2O)_{x-3} & \end{matrix}\right]$$

[1] As in Hückel's work, bonding lines are not drawn in the illustrated compounds.

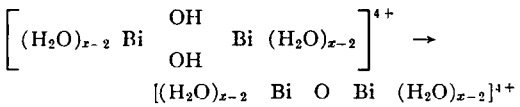
(*b*) The formation of polynuclear complexes. (In the course of this process, water is lost and the hydroxyl groups interact to link the bismuth atoms through "ol" bridges)



The following compound is illustrative of the further course of the reaction

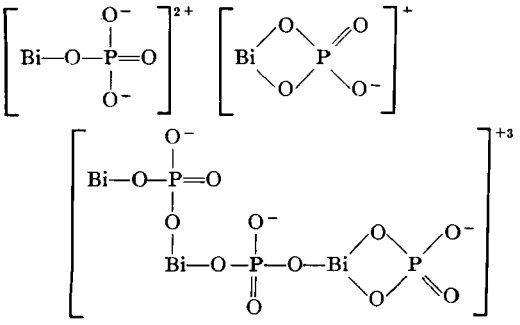


(*c*) An irreversible aging process. (The "ol" bridges are dehydrated and become "oxy" bridges.)

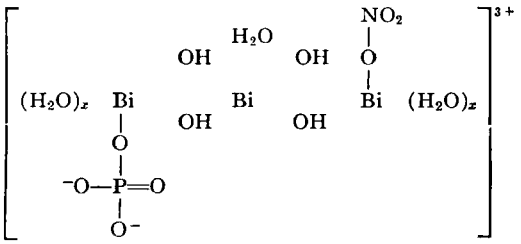


The above reactions seem reasonable when viewed in the light of data gathered by Kirk and Othmer (6). They report that a crystalline precipitate of the general formula, $Bi_2O_3 \cdot N_2O_5 \cdot 2H_2O$, is obtained when bismuth nitrate is added to water. With a 6% nitric acid solution, the composition shifts to $Bi_2O_3 \cdot N_2O_5 \cdot H_2O$. On further acidification, the precipitated material has the formula, $6Bi_2O_3 \cdot 5N_2O_5 \cdot 9H_2O$, and this in turn becomes $10Bi_2O_3 \cdot 9N_2O_5 \cdot 7H_2O$, when the suspension is heated to 70°. It can be seen that the transition from one state to the next involves a gradual loss of water and hydroxyl groups, which correlates with the reaction sequences indicated by Hückel's work.

Trivalent bismuth ions will also react with phosphate ions to produce the very slightly soluble bismuth phosphate. The latter compound is normally designated in the literature by its empirical formula, $BiPO_4$. However, structural requirements would indicate a crystal growth based on compounds of the following type



Reaction with phosphate ions at pH levels that would promote the formation of bismuth subnitrate would add to the complexity of the precipitate. The following suggests what might be found



EXPERIMENTAL

Method.—In this investigation, the bismuth subnitrate suspensions described by Haines and Martin (2, 3) were selected for study. Following the exploratory tests, the suspension concentration was raised to the 10% solids level, so that differences in suspension height would be more apparent and could be measured with greater relative accuracy. Settling rates were rapid enough to make this change practical. The experimental results were obtained and reported in accordance with the procedures discussed by Ward and Kammermeyer (8).

Equipment.—Settling data were obtained in 100-ml., glass-stoppered cylindrical graduates. Suspension heights could be measured within ±0.5-ml. accuracy, which was more than sufficient to establish a difference between the various suspension formulations.

Materials.—The same batch of a commercial grade of precipitated bismuth subnitrate (bismuth subnitrate N.F. supplied by Merck and Co., Inc., Rahway, N. J.) was used in the test series. The suspending medium was distilled water. Acid solutions were obtained by dilution from the concentrated commercial products, which were 70% nitric acid (supplied by E. I. du Pont de Nemours and Co., Wilmington, Del.) and 85% phosphoric acid (supplied by Mallinckrodt Chemical Works, St. Louis, Mo.). The two salts, monobasic potassium phosphate and trisodium phosphate, were of reagent grade (supplied by Merck and Co., Inc.).

Procedure.—The studies were run on a 10% bismuth subnitrate suspension, prepared by placing the bismuth subnitrate in a cylindrical, glass-stoppered graduate, adding about 30 ml. of distilled water, and shaking until a uniform suspension was obtained. A measured amount of dilute acid was added, the suspension again shaken, and the volume brought to 100 ml. by the further addition of distilled water. Where sodium citrate or trisodium phosphate were involved in the tests, they were introduced as concentrated solutions prior to the addition of the dilute acid solution.

The prepared suspensions were allowed to age for 8 hours, resuspended by shaking, then set aside for undisturbed settling. Readings on the suspension heights were taken approximately 48 hours later. Mechanical vibration or gentle agitation of any kind during the settling process had a marked effect upon the resultant height of the sediment. It is essential that settling take place of its own

accord. After the suspension heights had been recorded, the pH of each suspension medium was taken on a Beckman pH meter (glass electrode model H2), and the data were correlated with the suspension heights.

RESULTS

The bismuth subnitrate used in this series of experiments was found to suspend in water to give a pH of 3.05. A milky, uniform suspension was obtained, from which the bismuth subnitrate

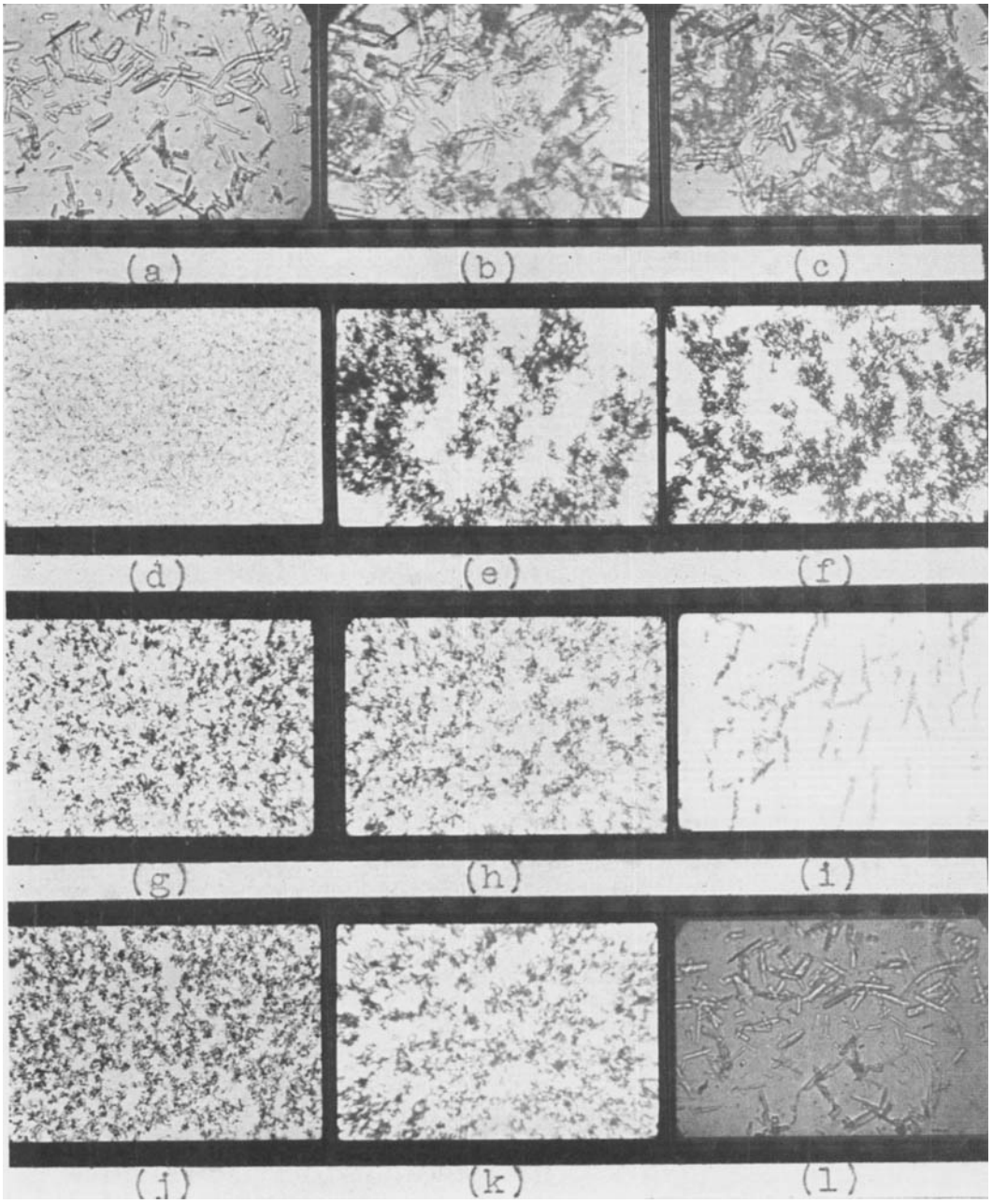


Fig. 1.—Key: (*a*) bismuth subnitrate in water (oil immersion); (*b*) bismuth subnitrate-flocculated (oil immersion); (*c*) bismuth subnitrate-flocculated (oil immersion); (*d*) bismuth subnitrate in water (low magnification); (*e*) bismuth subnitrate in nitric acid solution-pH 0.46 (low magnification); (*f*) bismuth subnitrate in phosphoric acid solution-pH 0.94 (low magnification); (*g*) bismuth subnitrate in trisodium phosphate solution (low magnification); (*h*) bismuth subnitrate with trisodium phosphate in nitric acid solution-pH 1.13 (low magnification); (*i*) bismuth subnitrate with trisodium phosphate in nitric acid solution-pH 0.86 (low magnification); (*j*) bismuth subnitrate with sodium citrate in nitric acid solution-pH 1.73 (low magnification); (*k*) bismuth subnitrate with sodium citrate in nitric acid solution-pH 0.65 (low magnification); (*l*) bismuth subnitrate in water (oil immersion). All suspensions were shaken vigorously prior to sampling.

particles slowly settled to form a dense precipitate. The supernatant liquid took several days to clear, which is indicative of the slowness of the settling rate. After all of the suspension had settled, the cylindrical graduate could be inverted, with the precipitate holding its shape and position at the bottom of the graduate. However, it was possible to resuspend the material easily if the compact precipitate was mechanically disrupted before the mixture was shaken. The appearance of the bismuth subnitrate crystals, microscopically, is shown in Fig. 1*a*, *d*, and *l*.

In the initial group of tests, various amounts of 7% nitric acid solution were added to a series of 10% bismuth subnitrate suspensions. The results are shown in Fig. 3. The bismuth subnitrate flocculated, and its relative suspension height more than doubled as the pH dropped below 1.0. A microscopic study showed that the individual bismuth subnitrate crystals were clumped into loose aggregates. Figure 1*e* compares the flocculated material against the original finely-divided suspension of crystals. The aggregates had a rapid settling rate and settled to leave a clear solution. It was found that the increase in suspension height was due to the voids between and within the aggregates as they slowly settled to form the precipitate. When the cylindrical graduate was carefully inverted, the precipitated material tended to hold its shape. In other words, there was some resistance to sediment flow under these conditions. The flocculated material could easily be resuspended by shaking. It should be emphasized, though, that the resuspended material was still in the aggregated condition and could not be shaken back to individual crystals, such as those seen in the untreated bismuth subnitrate suspension. To continue with this portion of the experimental work, it seemed of interest to study the acid-base response of the flocculation mechanism. Since the degree of flocculation was increased by hydrogen ion concentration, could the response be reversed by hydroxyl ions? A bismuth subnitrate suspension was treated with nitric acid until the pH had been reduced to approximately 0.8. The acid was then back-titrated with potassium hydroxide solution. A slight decrease in suspension height was seen, but certainly not a return to the original level. Whatever the changes induced by acid, it seemed they could not be reversed by a simple change in the pH.

Next, experiments were run in which the pH was followed as acids were added to bismuth subnitrate suspensions. Normal acid-base type neutralization curves might logically be expected, but this was not the

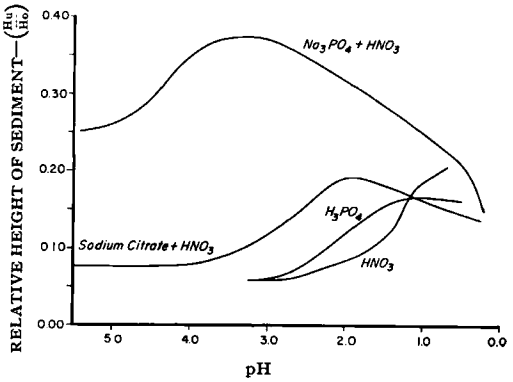


Fig. 2.—Composite plot of relative height of sediment *vs.* pH, demonstrating the relative activity of several flocculating agents.

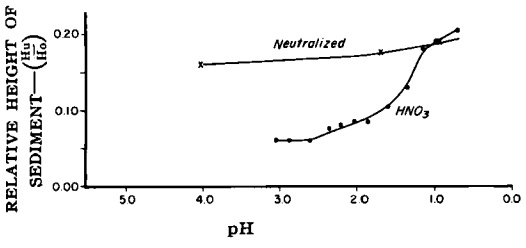


Fig. 3.—Relative height of sediment *vs.* pH for a bismuth subnitrate suspension flocculated by nitric acid. The acid was then neutralized with dilute potassium hydroxide solution.

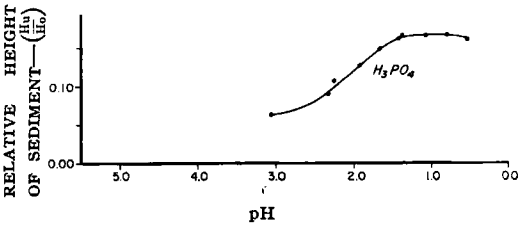


Fig. 4.—Relative height of sediment *vs.* pH for a bismuth subnitrate suspension flocculated by phosphoric acid.

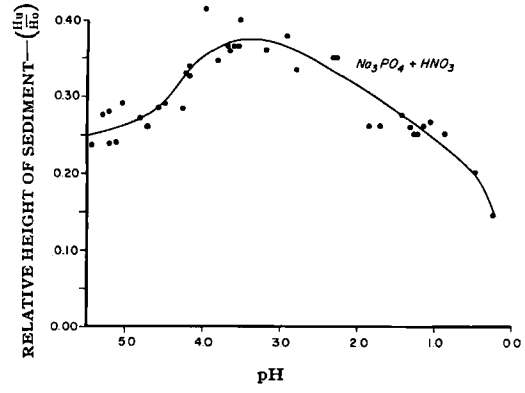


Fig. 5.—Relative height of sediment *vs.* pH for a bismuth subnitrate suspension flocculated by trisodium phosphate (4.72% w/v) and various amounts of nitric acid.

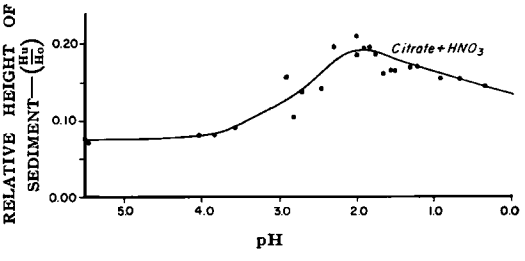


Fig. 6.—Relative height of sediment *vs.* pH for a bismuth subnitrate suspension flocculated by sodium citrate (9.80% w/v) and various amounts of nitric acid.

case. Titration of a 10% bismuth subnitrate suspension with either nitric acid or phosphoric acid gave a pH curve that was virtually identical with the pH curve obtained by titrating distilled water, if the distilled water was first adjusted to the initial pH of the bismuth subnitrate suspension. Although it is known that bismuth subnitrate will dissolve in strong mineral acids, quite concentrated acids are required. The pH must be well below 0.5 before a significant degree of dissolution is seen. The above observations suggested the existence of crystal surface changes as the result of the decrease in pH level. An equilibrium reaction appeared to be involved.

Figure 3 demonstrates the flocculation response as nitric acid was added to a bismuth subnitrate suspension. As the pH decreased, the relative suspension height showed a marked increase. The test was rerun, using phosphoric acid as the additive. Figure 4 gives the results of this test series, and it is apparent that the response was very similar to that obtained with nitric acid. The photographs of Fig. 1*d*, *e*, and *f* serve to illustrate the change that takes place as these acids are added. As hydrogen ion concentration increases, the individual crystals tend to clump together; the clumps act to give a bulkier, more readily resuspendable precipitate. In the lower pH range, the sediment would hold its shape when the graduate was cautiously inverted. This resistance to flow was more marked for the phosphoric acid series than for the nitric acid series. A definite "form" or "structure" was present, and it would have been possible to describe the sediment as a "soft cake." This correlates with the comments of Haines and Martin (2, 3). Simple shaking sufficed to resuspend the material.

The action of phosphate ion was further investigated through the addition of an excess of trisodium phosphate to a series of 10% bismuth subnitrate suspensions. Varying amounts of nitric acid were added, and data on relative suspension height *versus* pH were obtained and plotted as in Fig. 5. At all of the pH levels shown the relative suspension height was three or more times the height of the untreated material, and there was a maximum response between the pH levels 3.0 and 4.0. The flocculated material could easily be resuspended by shaking. However, here again, there was some evidence of a "structure" within the sediment. The experiment was run again, but sodium citrate was used in place of the trisodium phosphate. The results were somewhat comparable, as shown in Fig. 6. However, marked flocculation was not obtained until the pH had dropped below 3.0. The maximum fell between pH 1.5 and 2.5, and the relative suspension height decreased as pH decreased below 1.5. Microscopic studies on the test groups gave the typical photographs shown in Fig. 1. The graphical results have been summarized in the composite plot of Fig. 2.

DISCUSSION

Bismuth subnitrate is a crystalline material, occurring as transparent, elongated orthorhombic particles. Since the crystals themselves were identical in microscopic appearance throughout the course of each experiment, the variation in degree of flocculation must be because of differences in the composition of the molecular layers at the surface of the crystals. A crystal surface may vary in both the magnitude and sign of its electrostatic charges depending on the nature of the chemical groups residing in, or projecting from, its surface layer. The characteristics of the bismuth subnitrate crystal surface are influenced by the previous history of the crystal and by the chemical environment into which the crystal is placed.

The concept of a bonding between crystal surfaces is also required for an explanation of the experimental results. Such an attraction would be the end product of crystal surface conditions and could involve such things as electrostatic bonding, hydrogen bonding, or perhaps even van der Waals forces. When flocculating agents are present and the bismuth subnitrate crystals approach closely to one another, there is an attraction which culminates in a bonding of one crystal to the other. The bonding forces appear strong enough to hold the crystals in fixed relative positions during normal, undisturbed settling, but are weak enough to allow agitation to break the sediment into clumps of crystals, or for gentle vibration to reduce the relative suspension height by causing the suspension to settle more than it would under gravitational effects alone.

If one of the flocculated suspensions is shaken vigorously, the bismuth subnitrate is resuspended. However, it is not resuspended in the form of the individual crystals, but instead in the form of clumps of crystals, such as have been shown in our photographs taken through the microscope. The size of the clumps is determined by the force of the agitation used in resuspending the material, and by the number and strength of the bonding forces between the crystal surfaces. An additional factor is the variation in the density of the clumps. Our photographic work demonstrates an increase in density as the strength of the flocculating agent increases. The change in the density of the aggregates corresponds to an increase in the number or strength of bonds between the crystals.

In light of the above, the phenomenon of flocculation for a bismuth subnitrate suspension may be discussed as follows.

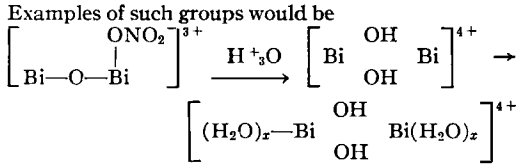
(*a*) When bismuth subnitrate crystals are produced, it appears that they have relatively few reactive points on their surfaces. It is presumed that the drying step tends to "seal in" some of the potentially active sites, as for example by the dehydration of "ol" bridges to "oxy" links.

$$\left[\begin{array}{ccc} & OH & \\ Bi & & Bi \\ & OH & \end{array}\right]^{4+} \rightarrow [Bi{-}O{-}Bi]^{4+} + H_2O$$

The drying step would leave the surface with fewer "ol" groups. As a result, the particles in suspension would have less tendency to clump, would settle independently under the influence of gravity, and would be piled randomly on top of each other in the sediment. As settling continues, the crystals would tend to realign themselves, filling the void spaces and bringing large areas of the crystal surfaces into close proximity with each other. After standing for several days, the bismuth subnitrate precipitate has the ability to retain its shape when the container is inverted. However, vigorous shaking, plus some mechanical dislodging of the sediment, will promptly resuspend the individual crystals. This may be viewed as a sediment in which there are bonds between the crystals, but in which the

bonds are relatively weak and few in number. They are effective in giving "form" or "structure" to the sediment, only because of the close contact between relatively large areas of the crystal surfaces. When the particles are in suspension, the bonding forces are so weak and few in number that they are ineffective in holding the crystals together.

(*b*) When nitric or phosphoric acid is added to a bismuth subnitrate suspension, reactive groups of various kinds will be formed on the crystal surfaces. Examples of such groups would be

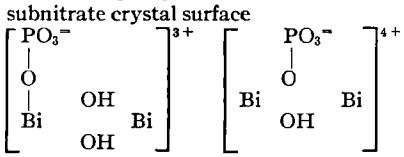


The higher the concentration of hydronium ion as flocculating agent, the greater the number of potential bonding sites that come into existence; thus the stronger is the total potential for forces of attraction and of bonding.

As small amounts of flocculating agents are introduced, there is a very loose clumping of the crystals in suspension. As these settle, the total effect of the bonding forces is weak enough to allow the crystals some freedom to realign themselves and thus to reduce the size of the voids in the sediment. However, the forces are active enough to prevent the complete realignment that was seen in the untreated bismuth subnitrate sediment.

At higher levels of flocculating agent, more and more bonding sites are created on the crystal surfaces. Their cumulative effect is strong enough to attract and bond tightly the individual crystals into compact, closely knit aggregates. When these aggregates settle, the crystals tend to be held in fixed relative positions and are thus unable to realign themselves to fill the voids in the sediment. Since the voids are present, the relative suspension height is correspondingly increased.

(*c*) The final system considered was that in which phosphate or citrate ions were introduced prior to the addition of the acid. Both anions give a somewhat similar response, so the discussion will be limited to the flocculating ability of the phosphate ion. Trisodium phosphate will react with the bismuth subnitrate crystal surface



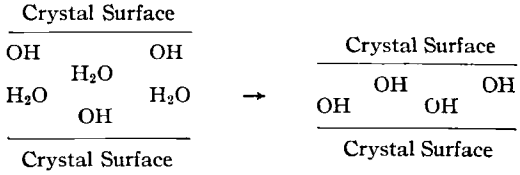
As has been said, some "ol" bridges and positive charges are present on the original crystal surface. It is for this reason that flocculation may be obtained at pH levels above the initial pH of the bismuth subnitrate suspension. In this region, however, there are relatively few of these "ol" bridges available for reaction, and the crystals tend to clump into very loose masses. As acid is added to this system, "oxy" links are converted to "ol" bridges and nitrate groups leave the crystal surface as ions. Additional bonding sites are thus formed.

The additional sites and the relatively high strength of the bonding forces developed by the presence of the phosphate groups give the loose, lacy clumps of bismuth subnitrate crystals a high degree of structural form. When they settle in the form of a precipitate, a vast amount of void space is enclosed. The sediment bulks to the unexpectedly large relative suspension heights noted between pH 3.0 and 2.0. As more and more acid is added, the number of bonding sites is increased, and the attractive forces tend to draw the crystals into clumps that are increasingly compact. The compactness eliminates the voids, and when the clumps settle, they enclose less void space even though their structural framework may be stronger than that obtained at the higher levels of pH. Thus the relative suspension height drops off from the maximum level and approaches the value given by the simple addition of phosphoric acid to a bismuth subnitrate suspension.

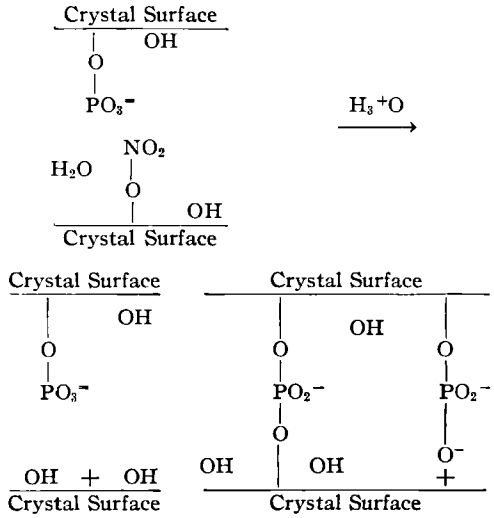
It should be emphasized that the response to the addition of phosphoric acid is different than that obtained when trisodium phosphate is added and followed by nitric acid. The fundamental difference lies in the characteristics of the clumps of crystals that are formed. In the phosphoric acid series, the crystals clump into relatively tight masses right from the start, and the compactness increases as additional acid is added. With trisodium phosphate, the initial clumping is very loose and does not become compact until sufficient nitric acid has been added to drop the pH below 2.0.

CONCLUSIONS

The flocculation of a bismuth subnitrate suspension has been explained in terms of a crystal surface phenomenon—one in which there is a mutual interaction between crystals that culminates in a bonding of one crystal to another. Two types of bonds seem to be the chief contributors, and these can be described on the basis of their relative strengths



Weak Bonds—"ol" Bridging



Strong Bonds—"Phosphate" Bridging

The weaker bonds are present to some extent in the original suspension, and the number of such bonds that can be formed increases as the concentration of hydrogen ion is increased. The stronger bonds are formed in the presence of phosphate ions, and again the number of potential bonds increases as the pH falls. An increase in hydrogen ion concentration tends to open additional reactive sites on the crystal surface.

The bonding between crystals gives rise to a clumping of crystals. As the clumps settle in the suspension medium, voids are formed both within and between the clumps. The differences in relative suspension height, from one system to the next, are a function of the number and size of the void spaces trapped within the sediment.

Where the bonds are weak and relatively few in number, a flocculated system fails to develop. The crystals sediment without the formation of voids, and the relative suspension height is at a low value. However, within the compacted sediment, the few weak bonds are able to exert sufficient bonding power to give the sediment some structural properties. Thus it shows some resistance to resuspension.

As the number of weak bonds increases, their total strength becomes sufficient to bond the crystals into a flocculated system. The crystals, although bound together, are able to adjust their relative positions as the precipitate settles. During this readjustment, the voids are decreased in size and an intermediate suspension height is obtained.

With a further increase in the number of weak bonds, the floc becomes quite compact. The bound crystals are capable of bridging the voids and of retaining them within the sediment. At this point, for the system involving weak inter-crystalline bonds, the relative suspension height reaches a maximum.

Where the bonds are strong but relatively few in number, as when sodium triphosphate is added to a bismuth subnitrate suspension, flocculation is seen. The floc settles, and the crystals are able to shift somewhat to eliminate a portion of the void space. However, a significant amount of void space is retained, and the sediment bulks to a substantial degree.

As more flocculating agent is added to form the stronger bonds, the density of the clumps increases slightly, but more importantly, the rigidity of the floc is increased. Thus when the material sediments, it is able to retain the voids and bulks to an extremely high relative suspension height.

SUMMARY

The bismuth subnitrate crystal surface is not easily described through structural formulas It is a highly solvated, three-dimensional complex involving hydrogen bonds, hydroxy links, ionic charges, and electrostatic bonds. The diagrams contained in this report should be considered as suggestive of the actual condition of the crystal surface rather than restrictive on the reader's concept of such a surface. Attention has been directed toward specific, isolated groups that are believed to be present.

When bismuth subnitrate is suspended in water, the crystal surface assumes a net positive charge because of the presence of ionic bismuth charges at the highly solvated surface. As phosphate is added to such a system, it reacts with both the "ol" groups and the available bismuth charges, first neutralizing the surface charge and then continuing to react further as more phosphate is added, until the surface charge has been reversed by a preponderance of phosphate ions bound to the surface. This concept finds support in the caking diagram of Martin (9), in which it was shown that the surface charge of suspended deflocculated bismuth subnitrate particles could be reversed by adding monobasic potassium phosphate to a suspension of bismuth subnitrate. The electrophoretic data was obtained in terms of particle movement, but was converted to zeta potential values when the caking diagram was drawn. Although zeta potential is a valid concept, its use in this situation tends to cloud the fact that the phosphate ions are reacting with the crystal surface—not gathering as an ionic atmosphere to shield the underlying charge on the particle surface.

A neutralization and reversal of net surface charge by chemical reaction of phosphate ions with the bismuth subnitrate crystal surface is the basic factor in the flocculation reaction under consideration in this report. It is important to recognize that a neutralization of net surface charge would yield a particle surface having "ol" groups, sites of positive charge, and sites of negative charge. The particle will not respond to electrophoresis at such an isoelectric point (a point at which there is a balance between negative and positive surface charges on the bismuth subnitrate particle). But if two crystal surfaces should come into close contact with each other, even at the isoelectric point, there would be ample opportunity for chemical or electrostatic interactions to occur between the surfaces and to end with the bonding of the two crystals in a flocculent state.

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Effect of Certain Tablet Formulation Factors on Dissolution Rate of the Active Ingredient I

Importance of Using Appropriate Agitation Intensities for *in Vitro* Dissolution Rate Measurements to Reflect *in Vivo* Conditions

By GERHARD LEVY

Evidence is presented to show that solid dosage forms are exposed to relatively low agitation intensities after oral administration, and that it is often mandatory to use similar mild agitation conditions for predictive *in vitro* dissolution tests. Based on considerations of the effect of stirring rate on boundary layer thickness, it is demonstrated that the effective surface area of heterogenous pellets (analogous in some respects to certain prolonged release dosage forms) may be different when such pellets are exposed to high and low agitation intensities, respectively. A proportionality between dissolution rate and the square root of stirring rate, demonstrated by others with rotating disks of inorganic salts, has been found to apply also to rotating disks of certain organic weak acids. It is shown that the ratio of dissolution rates of two or more drugs, when rates are determined by the rotating disk method, is independent of stirring rate, provided this proportionality or a similar more general relationship applies.

RECENT RECOGNITION that the availability for gastrointestinal absorption of drugs contained in compressed tablets is often reflected by *in vitro* dissolution rates and not by tablet disintegration times has stimulated the development and use of dissolution rate tests for compressed tablets (1–4). Ideally, the validity of a particular *in vitro* dissolution rate test for a given drug must be assured and proper interpretation of data made possible by demonstrating the correlation between *in vitro* and clinical data and by calibrating the former against the latter. The feasibility of such an approach has been demonstrated in a previous report from this laboratory (1). Based on physiologic considerations, we utilized a very low agitation intensity in our *in vitro* test (1, 5). In a study of the relation of *in vitro* dissolution rate to the rate of absorption of methylprednisolone implants in rats, Hamlin and co-workers have found that the *in vivo* results correlated only with those *in vitro* data that were obtained from dissolution tests utilizing very low agitation intensities (6). These workers were the first to point out the importance of stirring rate in dissolution rate studies of pharmaceuticals. The present communication deals with the development of meaningful dissolution rate tests for certain oral medication and includes the results of physiologic and physicochemical studies concerned with the effect of agitation intensity on the rate of dissolution of conventional compressed tablets and certain related dosage forms.

In this laboratory we have emphasized particularly the study of agitation conditions to which ingested tablets may be exposed in the stomach. One reason for this emphasis has been that solids may be retained in the stomach for a considerable length of time (7); therefore, the intestinal absorption of the ingested drug sometimes may be limited initially mainly to that portion which is dissolved in gastric fluids which flow into the intestine. Another reason for studying dissolution under conditions representative of gastric conditions has been the desirability (in many instances) that drugs reach intestinal absorption sites in absorbable (*i.e.*, dissolved) form, either because such drugs may be intrinsically slowly absorbed, or because they are absorbed only from the proximal region of the small intestine (26). Finally, part of our emphasis on gastric dissolution stems from our interest in the biopharmaceutics of salicylate drugs. Salicylates can be absorbed from the stomach, and their rapid dissolution and absorption is desirable in order to obtain a prompt analgesic effect. Perhaps more important is the prevention of serious mucosal erosion and bleeding because of aspirin solids that, on occasion, may lodge in the rugae of the gastric mucosa. Rapid dissolution of these solids will shorten the time of contact of the drug with the mucosa, and will tend to minimize the incidence and severity of mucosal damage (1, 8).

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EXPERIMENTAL

Radio-opaque Tablets.—The radio-opaque tablets

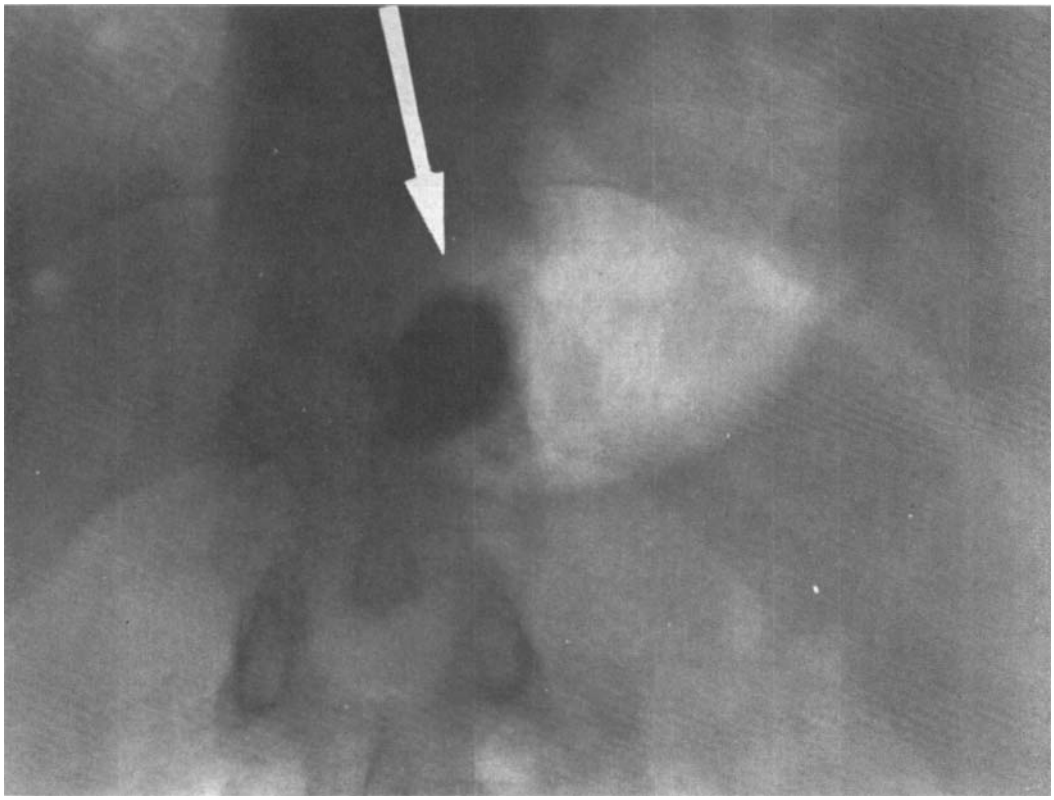
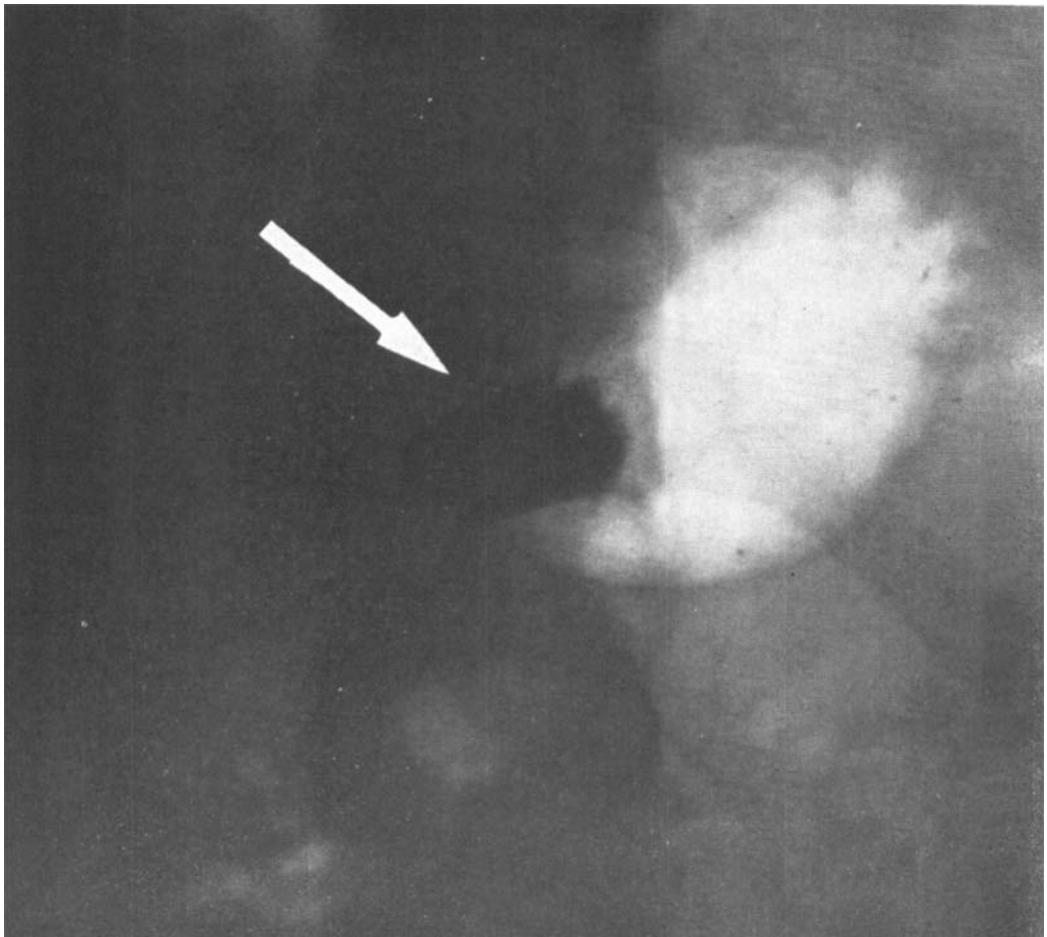


Fig. 1.—Radio-opaque tablet in human stomach 13 minutes after ingestion. Tablet is just beginning to disintegrate, as evidenced by the still noticeable tablet outline.

Fig. 2.—Radio-opaque tablet in human stomach 30 minutes after ingestion. Enlarged diameter of radio-opaque spot *vs.* Fig. 1 indicates that tablet has disintegrated.



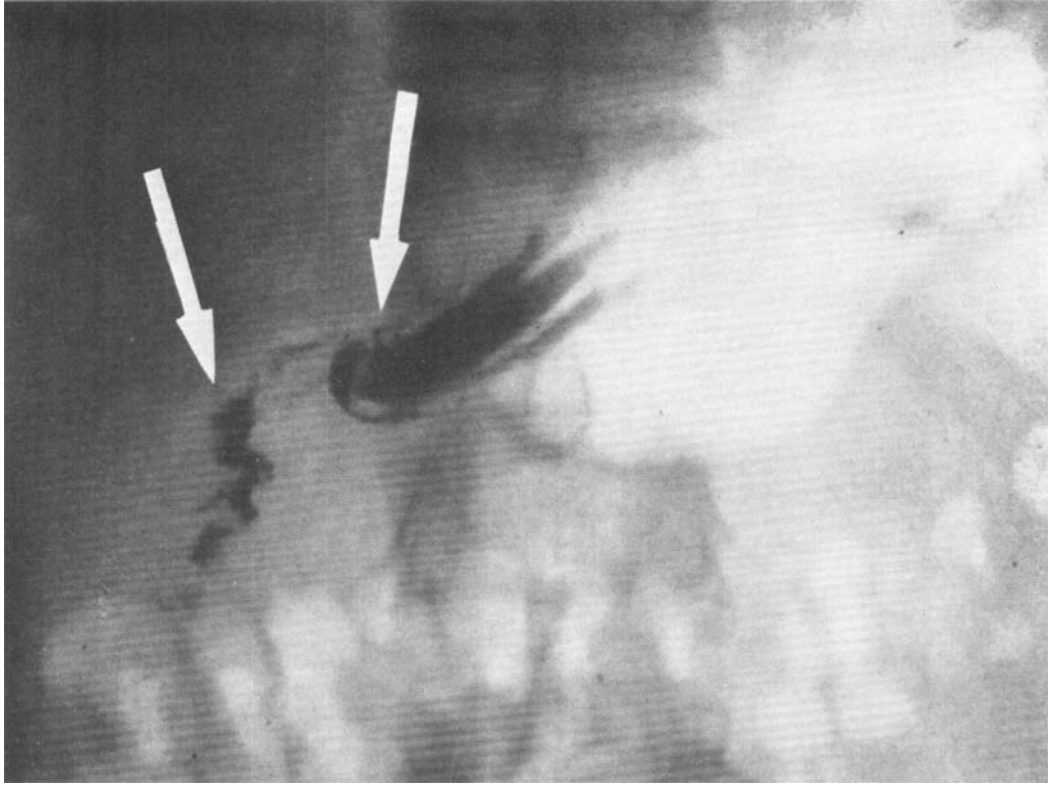
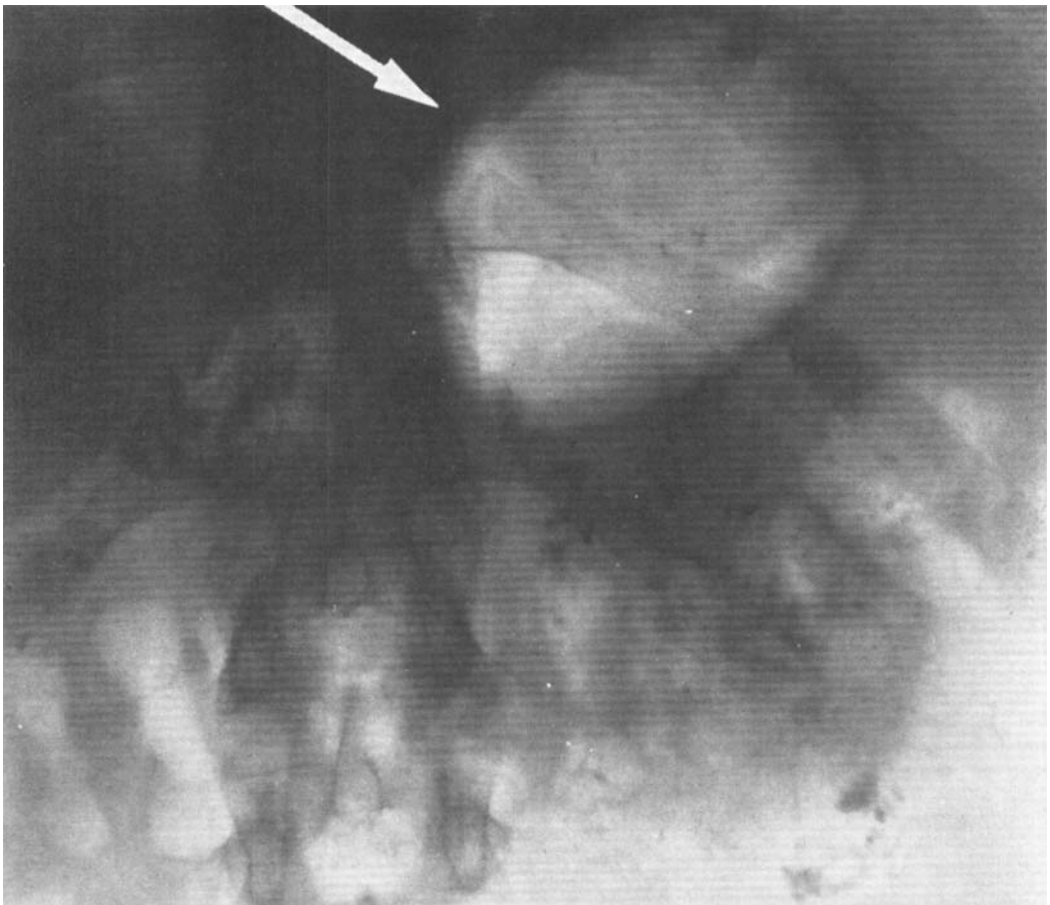


Fig. 3.—Radio-opaque tablet in human stomach 45 minutes after ingestion. Granules are passing through pylorus into small intestine. Note that granules are not dispersed throughout the stomach at any time.

Fig. 4.—Radio-opaque tablet solids in human intestine 60 minutes after ingestion of tablet. Arrow points to empty stomach. Granules are well dispersed in intestine and are apparent as very small radio-opaque spots.



used in this study were prepared by wet granulation of barium sulfate with starch paste and subsequent compression of the dried granulation to which dry starch had been added as a disintegrant.

Dissolution Rate Determinations.—Dissolution rates of conventional tablets were determined by the beaker method and by the oscillating tube method. The *beaker method* involves the use of a double-wall, 400-ml. capacity beaker and a propeller-type stirrer attached to an electronic-controlled stirring motor. The method has been described elsewhere in detail (5). The *oscillating tube method* makes use of a plexiglass cylinder (2.5 × 19.5 cm.) with a 100-mesh stainless steel wire screen on the bottom. The cylinder is attached to the basic unit of the U.S.P. tablet disintegration apparatus (where it replaces the standard basket-rack assembly). The cylinder is immersed in a beaker (placed in a constant temperature bath) containing 800 ml. 0.1 *N* HCl at 37°, the apparatus is set in motion, a tablet is dropped into the cylinder, and the medium is sampled at intervals by a fritted-glass immersion filter tube.

Intrinsic dissolution rate determinations and certain other experiments were carried out by the *rotating disk method* which has been described elsewhere (18). The *static disk method* is a modification of the rotating disk method. The stem of the pellet holder is inserted in a one-hole rubber stopper which serves as a closure for 12-dram plastic vials containing 25 ml. of 0.1 *N* HCl at 37°. The end of the pellet holder, and therefore the exposed face of the pellet, is thus immersed in the solvent. The entire assembly is placed in an incubator set at 37°. The pellet holder is removed from the solution at appropriate intervals (usually 1 or 2 hours), the plastic parts are dried carefully with tissue (without touching the pellet surface), and the assembly is placed in another vial which contains fresh solvent. After removal of the pellet assembly, an aliquot of the solution contained in the vial is assayed and, by appropriate calculations based on concentration and volume, the total amount of dissolved drug is established. This procedure yields data which may either be expressed in terms of an average dissolution rate (amount/area/time) or be plotted cumulatively as amount dissolved *versus* time.

Analytical Methods.—Salicylic acid was determined spectrophotometrically with a Beckman DU spectrophotometer. Solutions were diluted appropriately with 0.1 *N* HCl. Aspirin solutions were hydrolyzed by heating with sodium hydroxide, then analyzed for salicylic acid.

Microenvironmental pH was estimated by immersing the miniature electrodes of a pH meter (Leeds and Northrup model 7664) into a paste prepared from crushed tablets and 0.1 *N* HCl.

RESULTS AND DISCUSSION

Agitation Intensities Encountered in the Human Stomach.—X-ray motion pictures of the human stomach (viewed by the author) show that the agitation of gastric content during normal contractions is mild. It appears a valid assumption, therefore, that the particles from a disintegrated tablet are not dispersed throughout the stomach but rather remain as an aggregate. In support of this assumption of minimum agitation, it has been observed in animals that different foods, when ingested successively, remain in the stomach as different "layers" which do not intermix (9). Similar observations have been made recently in the human stomach by means of the fiberscope (10). Other pertinent reports are those of Ivy, *et al.*, who have remarked on the lack of significant mixing of material held in the fundus of the stomach (11), and several that deal with the regional differences in gastric pH—for example, Tomenius and Williams (12). Accordingly, we developed a dissolution rate test in which the agitation intensity is just sufficient to assure homogeneity of the medium for proper sampling. In this test the solids of the disintegrated tablet remain as an aggregate or "mound" in the center of the bottom of the beaker (5).

The essential correctness of our assumptions has been borne out by the excellent correlation of our *in vitro* test with *in vivo* data (1). However, it has been felt that more direct evidence could be obtained by a serial X-ray study of the behavior of a radiopaque tablet in the human stomach. Such tablets were formulated from barium sulfate in a manner assuring their rapid disintegration (U.S.P. disintegration time was only 3.5 minutes). Figures 1–4 are X-ray photographs taken 13, 30, 45, and 60 minutes after ingestion of a whole tablet with approximately 50 ml. of water.[1] The outline of the tablet was still apparent 13 minutes after ingestion, indicating that the tablet was just beginning to disintegrate. (The longer *in vivo* disintegration time suggests that the U.S.P. test may be too vigorous, or it may reflect the retarding effect of gastric mucous observed by Abbott, *et al.* (13)). Thirty minutes after ingestion, the tablet was totally disintegrated, but the solids remained in an aggregate approximately 2–3 cm. diam. (Fig. 2). The next picture (Fig. 3) was taken at 45 minutes, just as fluoroscopic examination showed that the solids were entering the small intestine. It can be seen that no dispersion of the solid particles occurred while they resided in the stomach. In the final picture of the series (Fig. 4), it is apparent that thorough dispersion of the particles did occur in the small intestine.

Subsequent to our study, Weiss, *et al.* (14), have reported the results of their gastroscopic observations of aspirin tablets in the human stomach; the results tend to confirm our findings. They noted during a period of 10 to 40 or 50 minutes after the ingestion of whole aspirin tablets that "frequently (the aspirin) appeared as a white gelatinous mass of granules adherent to the mucosa" and "in the more marked cases of bleeding, the pulverized mass of aspirin, coagulated blood, and mucus formed an adherent gelatinous layer of 10 to 15 mm. in diameter."

Differences in Comparative Dissolution Rates That May be Encountered When Using Different Agitation Conditions for Compressed Tablets.—The increase in dissolution rate of drugs contained in compressed tablets, when they are subjected to increasing agitation intensities, does not follow a simple mathematical relationship. A variety of effects, characteristic of each formulation, come into play and account for the need to use appropriate

[1] The author acknowledges gratefully the cooperation of Dr. Julian Ambrus, Roswell Park Memorial Institute, who took the X-ray photographs shown in this paper.

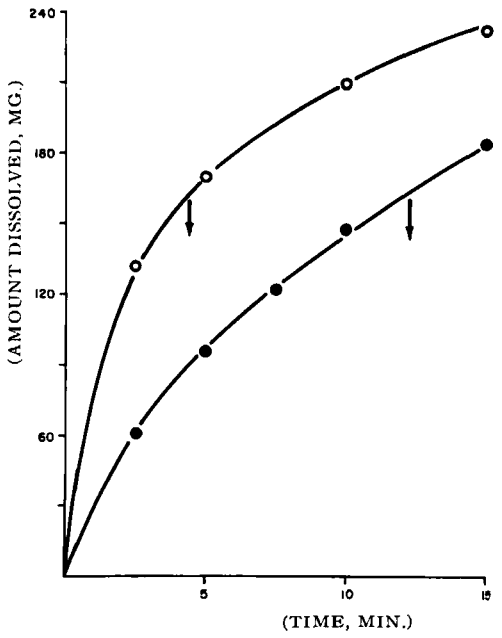


Fig. 5.—Dissolution of aspirin tablets (●) and "buffered" aspirin tablets (○) determined by the beaker method. Vertical arrows point to half-dissolution times.

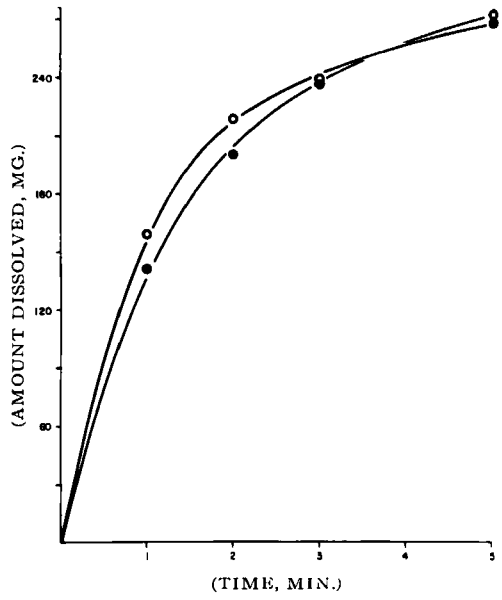


Fig. 6.—Dissolution of aspirin tablets (●) and "buffered" aspirin tablets (○) determined by the oscillating tube method.

agitation conditions for meaningful *in vitro* tests. When the solids of a disintegrated tablet form an aggregate or "mound," the effective surface area with respect to dissolution is primarily the surface of the mound, depending to some extent upon the porosity of the aggregate and the viscosity of the fluids in the interstices, among other factors. Under these conditions, the effect of particle size (specific surface area) on dissolution rate is considerably less than when the particles are dispersed throughout the medium by intensive agitation. This is evident from data presented in a following communication (15). The effect of other tablet components upon the dissolution rate of the active ingredient is also reduced under conditions of intensive agitation because of the greater physical separation of these components and the drug. On the other hand, such effects can be considerable in the microenvironment of a solid aggregate. They may result from high viscosity due to hydrocolloids and gums, lowering of interfacial tension by surfactants, other adsorption phenomena, changes in zeta potential associated with the presence of ionic species, chemical reactions because of high concentrations of potential reactants in the microenvironment, and particularly, changes in the pH of the microenvironment. These effects can directly or indirectly affect the primary drug particles and modify their rate of dissolution.

The importance of these considerations becomes evident from the following experiment. The dissolution rate of aspirin contained in (*a*) a proprietary product of plain aspirin in compressed tablets and (*b*) a proprietary product of aspirin and alkaline additives in compressed tablets was determined by two different methods. One was our previously described beaker method (5) which utilizes a very low agitation rate and permits the solid particles to remain on the bottom of the beaker as an aggregate. The other test was by the oscillating tube method which involves relatively high agitation intensities and causes the solid particles of the disintegrated tablets to be dispersed in the dissolution medium. The results of dissolution rate tests of the two tablet products, using the two different test methods, are shown in Figs. 5 and 6. Each point in the figures represents an average value obtained from four tablets.

Under the low agitation conditions of the beaker method, the tablets containing aspirin and alkaline additives dissolved much more rapidly than plain aspirin tablets. This is in accord with results of human absorption tests of these products conducted previously in our laboratory (1). The vertical arrows in Fig. 5 denote half-dissolution times—one comparative measure of dissolution rates. These times differed approximately threefold. At no time did the extreme values of the two types of tablets overlap. On the other hand, there was practically no difference in dissolution rates of the two tablet formulations when tested by the oscillating tube method (Fig. 6). The ranges of the respective values overlapped at all times, and half-dissolution times were essentially the same.

The difference in the results obtained by the two methods can be explained readily. In an aggregate of disintegrated tablet solids, the alkaline components of the "buffered" aspirin tablet caused an increase in the pH of the microenvironment from approximately pH 1 (0.1 *N* HCl) to about pH 5.6. This increase in pH results in a more rapid dissolution of aspirin particles (8).[2] The dissolution rate enhancing effect is absent when aspirin and alkaline components are physically separated by intensive

[2] A similar formulation approach can be used to increase the dissolution rate of other weak acids.

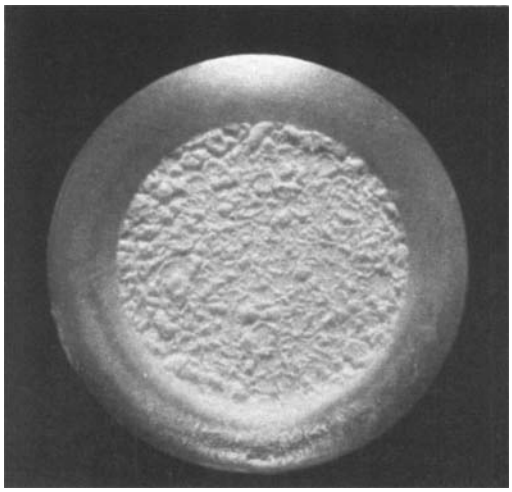


Fig. 7.—Pitted surface of a pellet made from pure salicylic acid granules and 3% magnesium stearate. Picture was taken after dissolution of 10 mg. salicylic acid from the pellet surface. (Pellet diameter, 1.27 cm.)

agitation, since the amount of alkaline components is insufficient to alter significantly the bulk pH of the medium. Thus it is evident that dissolution tests using agitation intensities sufficient to cause dispersion of particles would yield misleading results when used in the evaluation of the described type of pharmaceutical product.[3]

In this connection it is appropriate to comment upon the findings of Rubin, *et al.* (16), and Davison, *et al.* (17). These workers determined the pH of gastric fluids in humans after administration of a commercial "buffered" aspirin tablet product. They found no significant change in pH compared with control values. This should not be surprising on the basis of the quantities of alkaline components involved and the usual acidity and volume of gastric fluids. However, the significance of the cited observations can be misinterpreted easily. It must be recognized that the dissolution rate enhancing function of the alkaline components is because of their ability to raise the pH of the microenvironment, and that the absence of any change in bulk pH is of little pertinence in this respect.

In the course of studies of the effect of tablet formulation factors upon the dissolution rate of the active ingredient, we have also encountered differences in comparative dissolution rates as a function of agitation intensity in formulations that differed in starch content, granule size, and type of lubricant. These studies will be reported in subsequent communications.

Effect of Stirring Rate on the Rate of Dissolution from Irregular Surfaces of Heterogeneous Solids.—A phenomenon encountered in determinations of dissolution rate by the rotating disk method (18) may be pertinent to dissolution rate studies of nondisintegrating tablets containing several components, such as certain sustained-release pellets that consist of drug particles dispersed in a more slowly dissolving matrix. Pellets which were prepared by compressing a mixture of pure 20–40-mesh granules of salicylic acid and 3% magnesium stearate powder developed a rough pitted surface during dissolution. This effect became evident after dissolution of approximately 5 mg. of drug from the pellet, which had an exposed surface area of 1.27 cm.^{2} The surface pitting, which is clearly evident in a photograph of a representative pellet (Fig. 7), is because of the relatively rapid dissolution of salicylic acid granules, and the slower erosion of the hydrophobic magnesium stearate in the interstices between granules. In a dissolution rate determination where the pellets were rotated at 555 r.p.m. in 0.1 *N* HCl at 37°, an initial dissolution rate of 22.8 mg./hour/cm.^{2} was found. After 5 mg. of salicylic acid had dissolved and pits had formed on the pellet surface, the slope of the amount dissolved *versus* time plot increased until it yielded an apparent dissolution rate value of 34.6 mg./hour/cm.^{2} (19). This apparent 52% increase in dissolution rate can be attributed to the increase in surface area of the pellet as a result of pitting. When a similar test was conducted under static conditions (*i.e.*, the pellet was immersed in 0.1 *N* HCl but was not rotated), the dissolution rate was of course considerably lower (1.97 mg./hour/cm.^{2}), but the characteristic formation of surface pits did occur. However, pitting was not accompanied by an increase in apparent dissolution rate.

The observations described above can be explained on the basis of the changes in the thickness of the boundary layer as a function of stirring rate. The effective boundary layer thickness δ can be determined by (20)

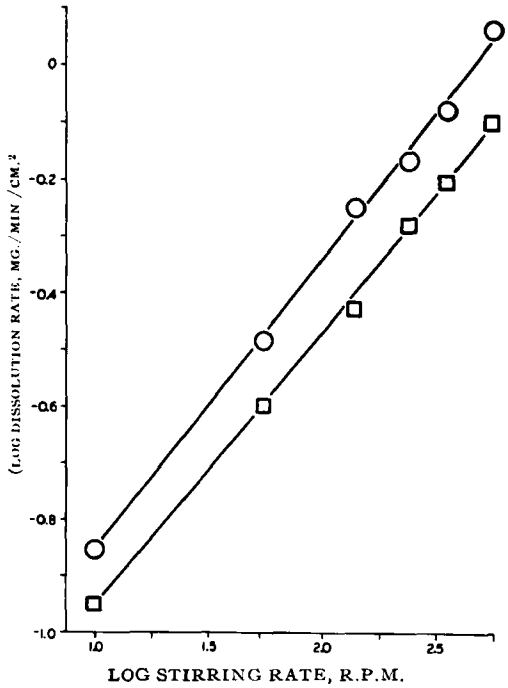


Fig. 8.—Plot of logarithm of dissolution rate *vs.* logarithm of stirring rate (spinning disk method). Key: (○) aspirin, (□) salicylic acid. (Dissolution rates of aspirin are expressed in terms of salicylic acid.)

[3] This should not detract from the usefulness of the oscillating tube method and similar methods to reflect the relative dissolution rates (particularly in the intestine) of many other types of drugs.

$$\delta = \frac{DSC_s}{R} \qquad \text{(Eq. 1)}$$

where D is the coefficient of diffusion, S is the surface area, C_s is the solubility, and R is the intrinsic dissolution rate. The diffusion coefficient of salicylic acid at 37°, calculated from Edwards' data (21), is 11.3×10^{-6} cm.²/second. This value is only slightly affected by concentration and may therefore be used as such. The solubility of salicylic acid in 0.1 N HCl at 37°, as determined in our laboratory, is 2.66 mg./ml. Thus, the boundary layer thickness for the pellets used in this study could be calculated by

$$\delta = \frac{(11.3 \times 10^{-6})(1.27)(2.66)}{R} \qquad \text{(Eq. 2)}$$

or

$$\delta = \frac{3.82 \times 10^{-5}}{R} \qquad \text{(Eq. 3)}$$

where δ is expressed in cm. and R is expressed in mg./second.[4]

The thickness of the boundary layer covering a pellet rotated at 555 r.p.m. is therefore

$$\frac{3.82 \times 10^{-5}}{6.34 \times 10^{-3}} = 0.0060 \text{ cm.}$$

while under static conditions, the boundary layer thickness is

$$\frac{3.82 \times 10^{-5}}{5.47 \times 10^{-4}} = 0.070 \text{ cm.}$$

The depth of the surface pits may be approximated in the following manner. The granules used to prepare the pellets were those which passed a 20-mesh screen but not a 40-mesh screen. This is equivalent to a granule diameter range of 0.042 to 0.084 cm. The maximum average depth of a surface pit may be considered to be equivalent to the average diameter of the granules and probably more realistically to one-half or two-thirds of this value. Since the pellets were compressed at very high pressure (20,000 lb.), it is likely that the granules were distorted in a manner which caused their elongation in a direction parallel to the pellet face and a decrease in diameter normal to the pellet face (22). Such a flattening would cause the depth of the pits on the pellet surface to be even smaller than indicated above. On the basis of all these considerations, the depth of the pits should, in all probability, be somewhat less than 0.04 cm. It is thus apparent that under static conditions (as contrasted with rapid agitation conditions) the surface irregularities would be effectively covered by the boundary layer, and the effective surface area of the pellet would not be increased by pitting. For this reason, there is no increase in the apparent rate of dissolution of the pellet during a static dissolution test. On the other hand, the thickness of the boundary layer covering the surface of a pellet rotating at 555 r.p.m. is insufficient to cover the pits which are formed during the dissolution process. Therefore, the effective surface area of the pellet increases as the smooth surface of the pellet becomes pitted; this results in an increase in apparent dissolution rate.

Although this may not be recognized readily upon initial consideration, the above can be pertinent to the evaluation of dissolution characteristics of certain nondisintegrating pellet-like dosage forms, such as some sustained-release tablets composed of a drug dispersed in a relatively hydrophobic matrix. These dosage forms are exposed to relatively mild agitation *in vivo*, and their effective surface area under these conditions may differ considerably from the effective surface area that is functional at higher stirring rates that may be used in an *in vitro* test. However, stirring rate should not be important if the rate of dissolution is limited by the rate of diffusion through a nondissolving coating or matrix, or if the boundary layer is not on the surface but within the tablet—as is the case with certain prolonged release tablets containing drug in a permanent plastic matrix.

Significance of Intrinsic Dissolution Rate Values Determined at Relatively High Stirring Rates.—For practical purposes, *intrinsic* dissolution rate determinations are frequently made at rather high stirring rates. In view of the emphasis placed in the present communication on the importance of using low stirring rates for dissolution rate tests of pharmaceutical dosage forms, the validity of biopharmaceutical interpretations based on intrinsic dissolution rate values obtained at high stirring rates (8, 23, 25) may be questioned. When the intrinsic dissolution rates of two substances differ by two or three orders of magnitude at high stirring rates, there should be little doubt that similar differences, at least qualitatively, should obtain at low stirring rates and under appropriate *in vivo* conditions. But what about differences in dissolution rates at high stirring rates that are only 50% or so in magnitude?

Cooper and Kingery (24) have recently presented the following equation which relates the diffusion-controlled dissolution rate from the surface of a rotating disk to the velocity of rotation (stirring rate),

$$j = 0.621\, D_o\, (V^*/D^*)^{1/3}\, (\omega/V_\infty)^{1/2}\, \Delta C \qquad \text{(Eq. 4)}$$

where j is the flux density of solute entering the solvent (dissolution rate per unit area), D_o is the diffusion coefficient of the solute in saturated solution, V^* is the effective viscosity in the immediate vicinity of the solid-liquid interface, D^* is the weighted average diffusion coefficient of the solute in the solvent (assuming that D varies with concentration), ω is the angular velocity (in radians per second) of the rotating disk, V_∞ is the bulk viscosity, and ΔC is the concentration differential, representing the difference between interface and bulk concentration. Under our usual experimental conditions, ΔC is maintained essentially constant and is equal to the solubility C_s of the solute in the solvent. In such cases,

$$j = K\omega^{1/2} \qquad \text{(Eq. 5)}$$

where

$$K = 0.621 D_o\, (V^*/D^*)^{1/3}\, (1/V_\infty)^{1/2} C_s$$

Equation 5 may also be written as

$$j = K_1\, (\text{r.p.m.})^{1/2} \qquad \text{(Eq. 6)}$$

[4] Certain complications associated with the application of Eq. 3 to heterogeneous pellets may cause the calculated δ values to be slightly too high (19). However, this will not affect the validity of the subsequent comments.

where

$$K_1 = 0.3236\ K$$

Expressed logarithmically, Eq. 6 yields

$$\log j = 0.5 \log (\text{r.p.m.}) + \log K_1 \quad \text{(Eq. 7)}$$

According to Eq. 7, a plot of the logarithm of dissolution rate (expressed here in mg./minute/cm.^{2}) *versus* the logarithm of stirring rate (expressed in r.p.m.) should yield a straight line with a slope of 0.5. This has been found by others to be the case with inorganic salts (20, 24).

We now present data to show (*a*) that the described relationship between dissolution rate and stirring rate holds for the apparatus used in our studies, and (*b*) that this relationship applies to compounds other than inorganic salts.

Using the rotating disk procedure (18), which is a modification and adaptation of the technique of Nelson (25), we determined intrinsic dissolution rates of aspirin and salicylic acid in 0.1 *N* HCl and 37° at stirring speeds ranging from 10 to 555 r.p.m. The data, appropriately plotted, are shown in Fig. 8. Each of the points in the figure represents from two to ten separate determinations. These points yield satisfactory straight lines which were fitted by the method of least squares. The slope of the line for salicylic acid is 0.492 and that for aspirin is 0.515. As is evident, the experimental slope values are very close to the theoretical value.[5]

It can be shown that the ratio of dissolution rates of two compounds is independent of stirring rate, provided the Cooper-Kingery equation or a similar, more general equation applies. Equation 7 may be rearranged to

$$\log (\text{r.p.m.}) = 2 \log j + K_2 \quad \text{(Eq. 8)}$$

where

$$K_2 = -2 \log K_1$$

On the basis of Eq. 8, at any given stirring rate

$$2 \log j_a + K_{2a} = 2 \log j_b + K_{2b} \quad \text{(Eq. 9)}$$

where the letter subscripts denote j and K_2 values for compounds *a* and *b*, respectively. Then

$$\log \frac{j_a}{j_b} = 1/2(K_{2b} - K_{2a}) \quad \text{(Eq. 10)}$$

and

$$\frac{j_a}{j_b} = 10^{1/2\,(K_{2b} - K_{2a})} = \text{constant} \quad \text{(Eq. 11)}$$

[5] This has also been found true for the dissolution of these weak acids in pH 8.0 Sorensen buffer solution.

In general, the ratio of the dissolution rates of two or more compounds will be independent of stirring rate as long as the respective dissolution rates increase with stirring rate according to the general equation

$$\text{dissolution rate} = (\text{constant})\,(\text{stirring rate})^a \quad \text{(Eq. 12)}$$

and the value of *a* is the same for each compound.

When these conditions are met, comparative dissolution rate data obtained at high stirring rates reflect properly the relative dissolution rates encountered at lower stirring rates. However, the observations by Hamlin and co-workers (6), and work currently in progress in this laboratory indicate that, in certain instances, the effect of stirring rate on dissolution rate cannot be described by either the Cooper-Kingery equation or by the general Eq. 12. In such cases it is often imperative to use appropriately low stirring intensities in intrinsic dissolution rate determinations for biopharmaceutical purposes.

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Effect of Certain Tablet Formulation Factors on Dissolution Rate of the Active Ingredient II

Granule Size, Starch Concentration, and Compression Pressure

By GERHARD LEVY, JANET M. ANTKOWIAK, JOSEPHINE A. PROCKNAL, and DONALD C. WHITE

The effect of various tablet formulation and processing factors on the rate of dissolution of the active ingredient of model tablets (prepared by double compression) was investigated. Dissolution rate increased with decreasing granule size (over the range 16–20 mesh to 60–80 mesh), but not strictly proportionally to the corresponding increase in the apparent surface area of the granules. Increasing starch content of granules (varied from 0 to 20 per cent) resulted in an increase in dissolution rate. Increasing precompression pressure (varied from 715 to 5720 Kg./cm.^{2}) caused an increase in dissolution rate. This was probably due to fracturing of the harder granules into smaller particles with greater specific surface area or bonding of the softer granules (prepared at lower slugging pressure) during their compression into tablets.

THE RATE of absorption and the physiologic availability of many drugs that are administered orally in solid form is a function of their rate of dissolution in gastrointestinal fluids (1–3). Certain tablet formulation and processing factors apparently affect the dissolution rate of drugs contained in tablets, since it has been found that generically identical tablet products made by different manufacturers exhibit significant differences in dissolution rate of the active ingredient (4). In a number of instances, poor tablet formulation has been shown to cause a significant reduction of physiologic availability of the active ingredient and impairment of clinical response (for example, see References 5–8).

There is frequently no correlation between tablet disintegration time and dissolution rate (2–4), or the nature of this correlation (if it exists) is variable and may be affected significantly by the nature of the formulation (9). Various aspects of tablet formulation and production have been the subject of extensive studies; but, unfortunately, disintegration tests rather than dissolution tests have been used to determine the effect of formulation and processing variables on the rate of release of the active ingredient. The need for information concerning the effect of tablet formulation factors on dissolution rate has led to studies that are the subject of this series of communications. The present communication deals with the effect of granule size, starch content of granules, and precompression (slugging) pressure on the rate of dissolution of the active ingredient of compressed tablets.

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EXPERIMENTAL

Preparation of Tablets.—Compressed tablets were made by double compression with a Carver hydraulic press, model B, using a conventional set of punches and die placed in a special holder. The punches were flat-faced and 1.27 cm. diam. Slugs were prepared from approximately 1 Gm. of powder and were subsequently broken into granules with a mortar and pestle. The granules were separated into the desired size fractions by sieving. Small amounts (sufficient for approximately 12 tablets) of the different granulations were mixed gently but thoroughly with other components of the formulation in small plastic vials. Quantities of mixture necessary to prepare a single tablet were weighed on an analytical balance and compressed into tablets. Preparation of mixtures and tablets took place on the same day on which the dissolution tests were made. The composition and other specifications of the experimental tablet preparations are listed in Table I.

Dissolution Rate Determinations.—Dissolution rates were determined by the beaker method, which has been described in detail (4). In one instance, the oscillating tube method (10) was used, except that the volume of dissolution medium was reduced to 300 ml.

GENERAL COMMENTS

In terms of physiologic realities, poor tablet formulation resulting in decreased dissolution rate of the active ingredient is most likely to affect the rate of gastrointestinal absorption of relatively poorly water-soluble weak acids. This is so because weak acids are generally well absorbed from the stomach (11) but dissolve much more slowly in acidic than in neutral or basic media (12, 13). On the other hand, weak bases dissolve much more rapidly in acidic than in basic media and generally are not absorbed to a significant extent from the stomach. Any retardation in dissolution of a weakly acidic drug may be reflected readily by slower absorption rate (2). A similar effect is less likely in the case of weak bases because of their more rapid dissolution

TABLE I.—SPECIFICATIONS FOR EXPERIMENTAL TABLETS

Formula Designation	Granules: Compn.[a]	Granules: Precompression Pressure[b]	Granules: Size[c]	Tablets: Compn.	Tablets: Compression Pressure[b]
A	SA, 80–100 mesh	2150	40–60	SA granules, 300 mg. Starch, 60 mg. Sodium lauryl sulfate, 9 mg.	715
B	SA, 80–100 mesh	2150	16–20 20–40 40–60 60–80	SA granules, 300 mg. Starch, 60 mg.	715
C	SA, 60–80 mesh Starch, 5% 10% 20%	1430	20–40	SA-starch granules, 300 mg. SA equiv. Starch, 15 mg.	715
D	SA, 60–80 mesh Starch, 15%	715 1430 2860 5730	20–40	SA-starch granules, 300 mg. SA equiv. Starch, 15 mg.	715

[a] SA = Salicylic acid. [b] Kg./cm.^{2} [c] U.S.P. mesh size.

in the acidic gastric fluids and because their absorption is restricted almost exclusively to the intestine. Therefore, gastric emptying rate rather than dissolution is likely to be the rate-limiting factor in the absorption of weak bases.[1]

These considerations have led to the use of a poorly water-soluble weak acid as the active ingredient in the tablet formulations prepared for this study. In view of the availability of an *in vitro* dissolution test method which has been shown to yield data which correlate with human absorption rate studies of salicylates (2), the weak acid salicylic acid was chosen. This compound has the additional advantages of stability and availability of a simple and direct method of analysis.

Studies described in this and the following communication (15) deal with certain formulation and processing variables encountered in the preparation of compressed tablets by double compression. This method of preparation does not involve the use of binders and liquids which could complicate considerably any reasonably well controlled study of variables associated with the preparation of tablets by wet granulation. However, many aspects of the results of the present study should be equally applicable to the preparation of tablets by wet granulation. It is recognized that the results obtained with tablets compressed with a hydraulic press may, in some instances, differ from those obtained with tablets compressed by means of a conventional tablet machine.

The effect on dissolution rate of some of the formulation variables studied may be mediated in some instances by a modification of granule and/or tablet disintegration rates. No attempt was made to evaluate the relative contribution of these as well as of certain microenvironmental factors.[2] The formulations, methods of preparation, and particularly the active ingredient, were chosen primarily for the purpose of obtaining model systems capable of yielding useful information. Accordingly, the choice of formulations was not restricted to those which might be of direct commercial pertinence.

RESULTS AND DISCUSSION

Aging Effects.—Preliminary experiments were carried out to determine the effect of aging on the dissolution rate of drug contained in compressed tablets because others have found that granulations prepared at different times may exhibit differences in properties (16). Salicylic acid granules were stored at room temperature in a desiccator containing Drierite; tablets were prepared from these granules after 1, 3, and 5 weeks of storage. Dissolution rates were determined on the same day on which the tablets were compressed. The results (shown in Fig. 1) indicate that dissolution rate changed significantly with granule age. To prevent significant bias or distortion of results because of

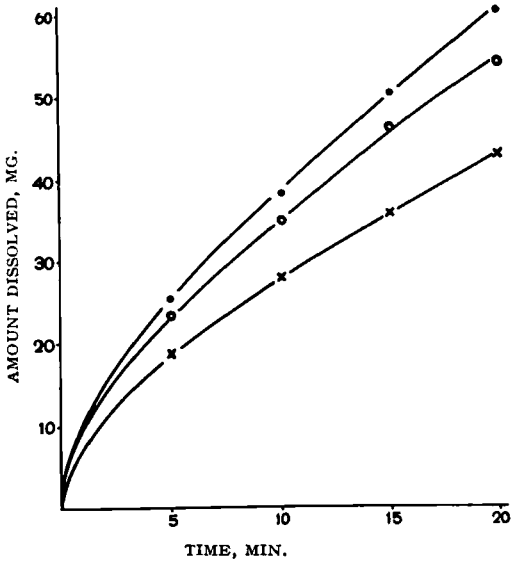

Fig. 1.—Effect of granule age on dissolution rate of salicylic acid contained in compressed tablets. Key: ●, one week; ○, 3 weeks; ×, 5-week-old granules. (Average of 10 tablets each, formula A.)

[1] Relatively rapid dissolution in acidic media and poor absorption from the stomach may be the reasons why the rate of absorption of tetracycline hydrochloride has been found to be independent of particle size (14).

[2] The reader may refer to the first paper of this series (10) for an explanation and description of microenvironmental factors.

aging effects, all subsequent experiments were conducted in the form of a latin-square design; dissolution tests for a given experiment were carried out as rapidly as possible to make all measurements within a few days. This also reduced possible bias because of effects resulting from fluctuations of temperature and humidity.

Effect of Granule Size.—Figures 2 and 3 show dissolution rate data for tablets made from 16–20-mesh, 20–40-mesh, 40–60-mesh, and 60–80-mesh granules, respectively. It is evident that the dissolution rate increases with decreasing granule size. However, this increase is not strictly proportional to the increase in the apparent surface area of the granules (Table II). The surface area ratios listed in Table II were calculated on the assumption that the granules were spherical and had smooth surfaces. The ratios should be approximately correct even if these assumptions do not obtain, because shape factor and surface roughness should be about the same for granules of all sizes used.[3] Dissolution rate ratios could be calculated from initial dissolution rates because these were approximately constant until 15% of the drug content of a tablet had dissolved. The increase in dissolution rate from

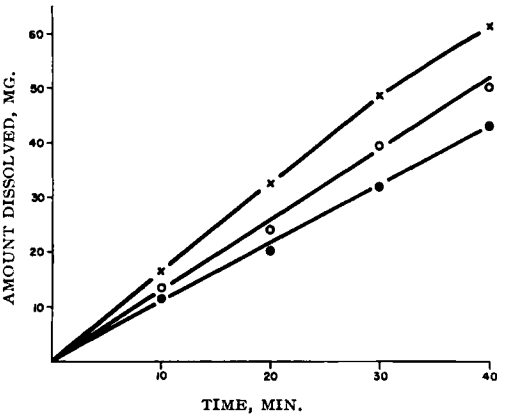

Fig. 2.—Effect of granule size on dissolution rate of salicylic acid contained in compressed tablets. Key: ●, 16–20 mesh; O, 20–40 mesh; ×, 40–60 mesh granules. (Average of 10 tablets each, formula B.)

tablets containing 20–40-mesh and 40–60-mesh granules (compared with tablets made from 16–20-mesh granules) was not so great as the increase in apparent surface area. This is because the disintegrated tablet particles remain as an aggregate on the bottom of the beaker (due to the low intensity of agitation), and the surface area of drug exposed to the solvent is primarily the surface of the aggregate. This effect has been explained in greater detail in the first paper of this series (10).

The much greater dissolution rate of tablets prepared from the smallest granules (60–80 mesh) is because these granules are small enough to be dispersed somewhat in the medium despite the low intensity of agitation. This permits moving solvent to come in contact with a greater portion of the potentially available surface. The granule size which would show such an effect in the human stomach

[3] It has been shown recently that the shape factor for 20–30-mesh anisometric crystals of salicylamide could be assumed to remain constant at least until about 30% was dissolved (17).

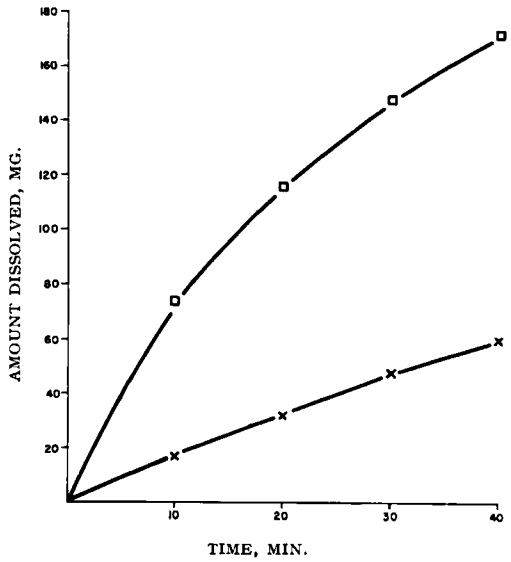

Fig. 3.—Effect of granule size on dissolution rate of salicylic acid contained in compressed tablets. Key: ×, 40–60 mesh; □, 60–80-mesh granules. (Average of 10 tablets each, formula B.)

would depend on the specific gravity of the granules and on gastric motility and cannot be estimated readily except by *in vivo* X-ray studies.

Effect of Starch Content of Granules.—Increasing the starch content of granules from 5 to 20% resulted in an increase in the dissolution rate of salicylic acid (Fig. 4), probably because of more rapid and thorough disintegration of the granules. (It is known that tablets disintegrate more rapidly when the starch content is increased (18, 19).) In this instance, dissolution rate determinations were also made with the oscillating tube apparatus (Fig. 5), and the results, although qualitatively similar, differ somewhat from those obtained by the beaker method. This illustrates again the need to use appropriate methodology for dissolution rate studies of compressed tablets (10.)

Effect of Granule Compression Pressure.—Tablets were prepared from granules made from slugs compressed at pressures ranging from 715 to 5730 Kg./cm.[2] As shown in Fig. 6, dissolution rates increased with increasing precompression pressure. This may be due to fracturing of drug particles at higher slugging pressure, yielding smaller primary particles. Such an effect was observed by Higuchi, *et al.*, with aspirin (19). Fragmentation

TABLE II.—ESTIMATED RATIOS OF SURFACE AREAS AND RATIOS OF INITIAL DISSOLUTION RATES OF SALICYLIC ACID CONTAINED IN COMPRESSED TABLETS[a] AS A FUNCTION OF GRANULE SIZE

Granule Size, U.S.P. mesh	Av. Granule Diam. cm.	Surface Areas,[b] Estimated Ratios	Initial Dissolution Rates, Ratios
16–20	0.102	1.0	1.0
20–40	0.063	1.6	1.2
40–60	0.034	3.0	1.5
60–80	0.021	4.9	6.9

[a] Tablet formula B. [b] Based on assumed sphericity of granules.

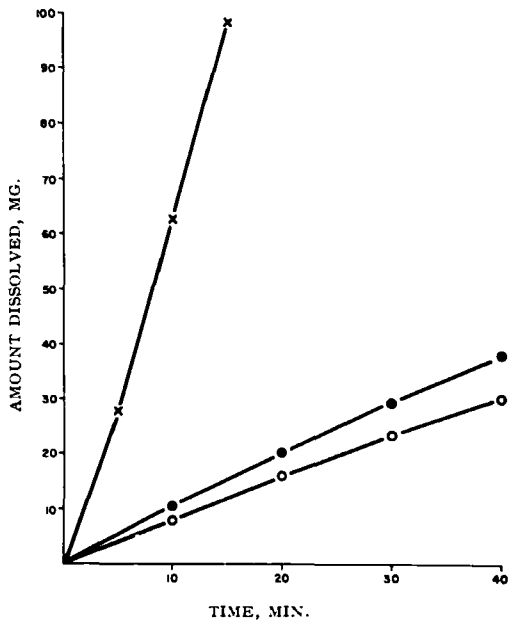

Fig. 4.—Effect of starch content of granules on dissolution rate of salicylic acid contained in compressed tablets. Key: O, 5%; ●, 10%; ×, 20% starch in granules. (Average of five tablets each, formula C.)

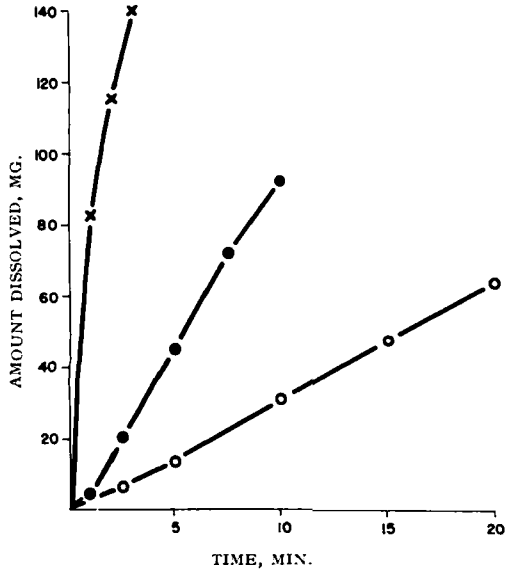

Fig. 5.—Effect of starch content of granules on dissolution rate of salicylic acid contained in compressed tablets, determined by the oscillating tube method. (Symbols, etc., same as in Fig. 4.)

of the more highly compressed granules during subsequent tableting may also occur. Finally, the softer granules obtained at lower precompression pressures are more likely to undergo bonding during tableting, and thus yield larger granules. Visual observations suggest that the latter effect is operative, but this does not exclude a possible contribution because of one or both of the other effects.

It has been found that aspirin administered in

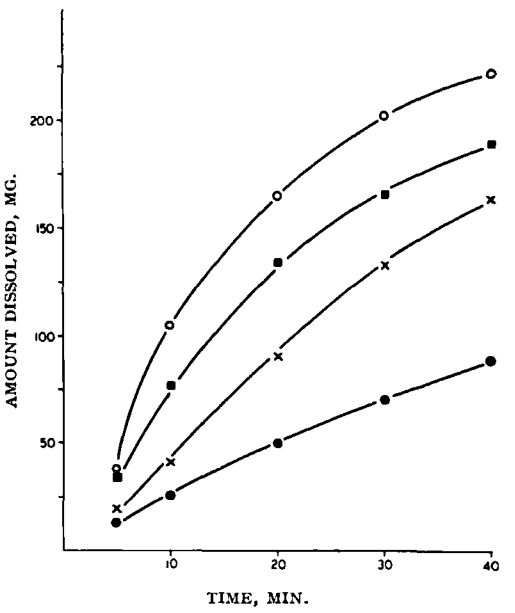

Fig. 6.—Effect of precompression pressure on dissolution rate of salicylic acid contained in compressed tablets. Key: ●, 715 Kg.; ×, 1430 Kg.; ■, 2860 Kg.; O, 5730 Kg. pressure per cm.^{2} (Average of five tablets each, formula D.)

tablets made at very high compression pressure was more rapidly absorbed by humans than aspirin administered in tablets compressed at lower pressure (20); this could be because of the same effects reported here.

Although the data shown in Figs. 4 and 6 represent separate experiments carried out at different times, the dissolution rate curve in Fig. 6 for the tablets made from granules prepared at 1430 Kg./cm.^{2} pressure (and containing 15% starch) falls between the curves for tablets made from granules that were also compressed at 1430 Kg./cm.^{2} and contain 10 and 20% of starch, respectively (as shown in Fig. 4).

CONCLUSIONS

Results of this study indicate that some of the more important tablet formulation and processing variables may affect the dissolution rate of the active ingredient. The magnitude of these effects must be determined individually for each tablet product, and their significance with respect to the rate of gastrointestinal absorption and the physiologic availability of the active ingredients must be assessed on the basis of several additional considerations (10, 21). The results of the present investigation should only be interpreted as indicative of the general nature of the effects on dissolution rate of the tablet formulation and processing factors studied.

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Nonclassical Antimetabolites XIV

Some Factors in Design of Irreversible Inhibitors Effective at Low Concentration

By B. R. BAKER, R. P. PATEL, and PRABODH I. ALMAULA

5-(Bromoacetyl)salicylic acid (XII) irreversibly inhibits glutamic dehydrogenase and lactic dehydrogenase about as well as the standard exoalkylating agent, 4-(iodoacetamido)salicylic acid, at one-tenth the concentration of the standard; most of this increased reactivity is because of the higher reactivity of the halogen in XII. Three of the four iodoacetamido compounds that reversibly bound to lactic dehydrogenase more tightly than the standard gave irreversible inhibition at 10–20 per cent the concentration necessary for the standard. Three carbophenoxyamino heterocyclic acids showed no irreversible inhibition of lactic dehydrogenase, but were effective irreversible inhibitors of glutamic dehydrogenase at one-tenth the concentration of the standard compound.

THE CONCEPT (2) that a properly designed inhibitor can reversibly complex with the active site of an enzyme, then become irreversibly bound within the complex by alkylation adjacent to the active site (exo-alkylation) has had strong experimental support (3, 4). Similar observations have been independently and subsequently made in the area of hapten immunochemistry (5) and with chymotrypsin (6).

The exo-alkylation phenomenon is illustrated (4) by Eq. 1. In the experimental evidence for this phenomenon, it was shown that the rate of irreversible reaction between the inhibitor AB and the enzyme, to form the irreversible complex, II, was dependent upon the concentration of reversible complex, I; that is, the rate of inactivation could not be increased once the enzyme was saturated with the inhibitor. The amount of reversible complex, I, can be calculated from the measurable dissociation constant, K_I, of the enzyme-inhibitor complex.

$$E\!\!<^{H} + AB \rightleftharpoons E\!\!<^{H} \cdots AB \rightarrow \underset{\text{I}}{} \quad E\!\!\overset{\frown}{\cdots A} + BH \quad \text{(Eq. 1)}$$
II

Of obvious importance to chemotherapy is that a sufficient intracellular concentration of an inhibitor must be obtained to effect the target enzyme site of an invading cell. In order to overcome an insufficient intracellular concentration of inhibitor, it would be necessary to find inhibitors that will operate at lower concentrations. There are two ways in which exo-alkylating irreversible inhibitors effective at lower concentrations could be obtained. First, a compound that can saturate the enzyme at a lower concentration (smaller K_I) will operate equally as effectively as an agent that requires a higher concentration to saturate the enzyme (larger K_I); second, if a more active alkylating group, B, in structure I is employed, the rate of inactivation of the enzyme should be the same at proportionally lower inhibitor concentrations. The design, synthesis, and evaluation of both types are the subject of this paper.

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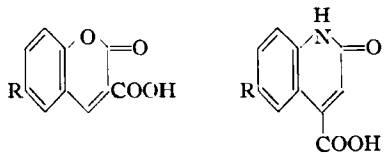
TABLE I.—SYNTHESIS OF CARBOPHENOXYAMINO AND IODOACETAMIDO HETEROCYCLIC CARBOXYLIC ACIDS

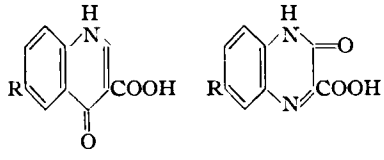
Compd. No.[a]	Method[b]	Yield, %	M.p., °C.	Anal. Calcd. C	H	N	Found C	H	N
III*d*	A	78	215–216[c]	38.6	2.16	3.75	38.8	2.30	3.84
III*e*	A	52	218–220[d]	62.9	3.42	4.32	62.9	3.51	4.32
IV*d*	C	33	above 300[e]	38.8	2.44	7.55	38.7	2.64	7.31
IV*e*	B	43	above 300[e]	63.1	3.73	8.65	62.9	3.90	8.71
V*d*	E	61	above 300[f]	38.8	2.44	7.55	39.0	2.61	7.48
V*e*	E	77	above 300[e]	63.1	3.73	8.65	62.8	3.82	8.42
VI*d*	D	43	above 300[g]	35.3	2.16	11.3	35.2	2.37	11.3
VI*e*	C[h]	31	above 300[e]	59.2	3.42	12.9	58.9	3.59	13.1

[a] Each compound had the expected infrared spectrum. [b] See *Experimental*. [c] Recrystallized from 95% ethanol. [d] Recrystallized from ethyl acetate. [e] Recrystallized from absolute ethanol-petroleum ether. [f] Recrystallized from 2-methoxyethanol-water. [g] Recrystallized from ethanol-water. [h] Although the sodium salt of VI*c* was insoluble, *Method C* worked better than *Method D*.

The prototype of exo-alkylating irreversible inhibitor was 4-(iodoacetamido)salicylic acid (3,



III IV



V VI

a series, R = H
b series, R = NO_2
c series, R = NH_2

d series, R = $ICH_2\overset{\overset{O}{\|}}{C}NH-$
e series, R = $C_6H_5O\overset{\overset{O}{\|}}{C}NH-$

4). A number of compounds have been observed (7) to give lower I_{50} values[1] for LDH and GDH[2] than the prototype, salicylic acid (8). For example, salicylate had I_{50} = 19 on LDH, coumarin-3-carboxylic acid (III*a*) had I_{50} = 1.7, 2-hydroxycinchoninic acid (IV*a*) had I_{50} = 0.18, and 1,6,7-trimethyl-2-quinolone-3-carboxylic acid had I_{50} = 0.20; thus these compounds had K_I values of 0.1 to 0.01 of the original salicylic acid. The structural feature necessary for inhibition of LDH and GDH that these compounds had in common was the 1,2- or 1,3- relationship of oxo (or hydroxyl) and carboxyl groups (7).

Two other compounds that should reversibly inhibit LDH and GDH are 4-hydroxyquinoline-3-carboxylic acid (V*a*) and 2-hydroxyquinoxaline-3-carboxylic acid (VI*a*). The latter was resynthesized (10) and showed reversible inhibition of LDH and GDH; however, the I_{50} concentration could not be reached because of the amount of absorption of light at 340 mμ by the compound. It was expected that the carbophenoxyamino (9, 11) and iodoacetamido derivatives of III–VI should show lower K_I values than the standard compound, 4-(iodoacetamido)salicylic acid (3, 4) and therefore could potentially operate as exo-alkylating agents at lower concentrations than that used for the standard compound.

EXPERIMENTAL

Reagents.—α-Ketoglutaric acid, sodium pyruvate, DPN, LDH, and GDH were purchased from Sigma Chemical Co. and from Nutritional Biochemical Corporation; LDH was the crystalline enzyme isolated from rabbit skeletal muscle, and GDH the crystalline enzyme isolated from mammalian liver.

Determination of I_{50} Values.[1]—The procedure employed for LDH was the same as that previously described (7, 8), except that 0.05 *M* Tris buffer (pH 7.4) and 1 m*M* pyruvate were employed (12). The I_{50} values for GDH were determined in the same fashion as for LDH using cell concentrations of 1 m*M* α-ketoglutarate and 75 m*M* ammonium sulfate in 0.05 *M* Tris buffer (pH 7.4). In some cases with colored compounds (for example compound IV*d*), the I_{50} concentration could not be reached because of insufficient light transmission at 340 mμ for the kinetic study. In such cases, the highest concentration possible was used that allowed full light transmission at 340 mμ and the I_{21} or I_{30} was noted; these I_x values could be converted to the K_I values in Table II by the calculations shown in the section on *Results*.

Enzyme Inactivation Procedure.—The LDH inactivation procedure (4), as later modified (12), was employed. The GDH incubations were run as previously described (4). In all incubations for determination of irreversible inhibition, three incubation solutions were run simultaneously and were made from the same master enzyme-DPNH solution (4): (*a*) a solution of the test compound, (*b*) a standard of 2 m*M* 4-ISA, and (*c*) an enzyme control with no inhibitor. All runs were duplicated at least twice. An occasional bad run was readily elimi-

[1] The I_{50} is defined as the concentration of inhibitor (I) that will give 50% inhibition of enzymic reaction in the presence of 1 m*M* concentration of substrate *S* (8). The I_{50} and the enzyme-inhibitor (EI) dissociation constant K_I can be interrelated (9) by the dissociation constant, K_m, of the enzyme-substrate complex with use of the formula

$$K_I = K_m \times I/S = K_m \times I_{50}$$

[2] Abbreviations: LDH, lactic dehydrogenase; GDH, glutamic dehydrogenase; DPNH, reduced diphosphopyridine nucleotide; 4-ISA, 4-(iodoacetamido)salicylic acid.

TABLE II.—RELATIVE EFFECTS OF INHIBITORS ON LDH AND GDH

Compd. No.	Thiosulfate Reactivity	Enzyme	$K_I \times 10^4$	Inactivation, Rate	Incubation Concn.[a]
4-ISA	1.0[c]	LDH	17[b]	1.0[c]	2.0
		GDH	8.5[b]	1.0[c]	2.0
XII	19	LDH	13	1.2, 1.2	0.2
		GDH	11	0.65, 0.63	0.2
III*d*	1.13	LDH	3.1[d]	0.45, 0.59	0.4
IV*d*	0.63	LDH	3.2[e]	0.56, 0.46	0.2
V*d*	...[g]	LDH	0.87	0, 0	0.2
VI*d*	...[g]	LDH	1.4[d]	0.76, 0.83	0.2
III*e*	...	LDH	1.9	0, 0, 0	0.4
		GDH	2.0	0.62, 0.58	0.2
IV*e*	...	LDH	2.4	0, 0, 0	0.4
		GDH	2.4	1.0, 1.3[f]	0.2
V*e*	...	LDH	1.1[d]	0, 0	0.13[h]
		GDH	2.3[d]	0, 0	0.13[h]
VI*e*[i]	...	...	...	...	...

[a] Millimolar concentration of inhibitor; 2 m*M* 4-ISA used for comparison (see *Methods*). [b] These values calculated from the I_{50} (7) (see *Results*) are slightly different from the K_I values obtained by Lineweaver-Burk plots (4) and agree within experimental error for both methods (9). [c] Arbitrary values for relative purposes; these are not the same absolute value for LDH and GDH. [d] Calculated from I_{50} value. [e] Calculated from I_{20} value. [f] This compound showed a rate saturation effect (4, 12) in two runs comparing 0.12 and 0.24 m*M* and comparing 0.15 and 0.30 m*M* (Fig. 3) with ratios of 1.4 and 1.1, respectively; calcd. ratios from Eq. 4: 1.5 and 1.4. [g] Sodium salt too insoluble to determine, but all N-aryl iodoacetamides previously measured have shown 1.0 ± 0.4. [h] Maximum concentration obtainable at 0° in Tris buffer (pH 7.4) was 0.20 m*M*. [i] Solubility less than 0.2 m*M* at 0° in Tris buffer (pH 7.4) and therefore K_I and irreversible inhibition not determined.

nated if the control or standard did not behave properly.

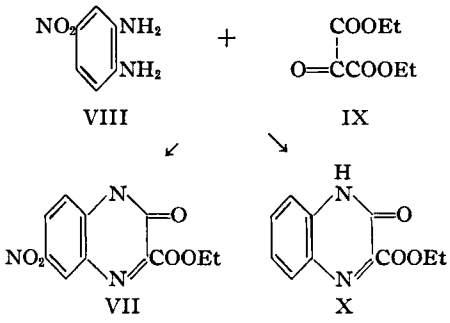
Reactivity of Halogen.—The method for determination of the relative halogen reactivity previously employed (4) was not satisfactory for the compounds listed in Table II. Whereas 4-ISA had a half reaction time with thiosulfate of about 60 minutes at 0°, 5-(bromoacetyl)salicylic acid (XII) had half reacted at this temperature and concentration in about 3 minutes. Satisfactory kinetics were obtained when the reaction and the blank were diluted tenfold; XII reacted 19 times faster than 4-ISA (Table II).

The other compounds in Table II (III*d*–VI*d*) formed insoluble sodium salts at 0° with the previous method (4). The method was modified by allowing the reaction to proceed at 24°, but the increased rate in reaction was compensated by running the kinetics at a fourfold dilution of that previously used (4). The standard compound, 4-ISA was rerun under these warmer, more dilute conditions; comparisons are listed in Table II where 4-ISA is given an arbitrary rate of 1.0.

CHEMISTRY

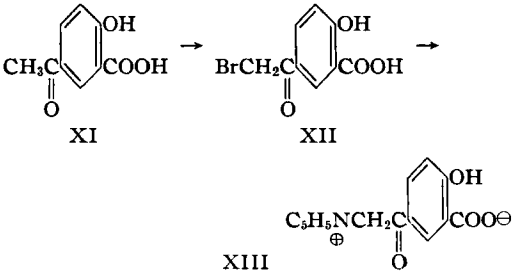
Methods.—All of the potential irreversible inhibitors were synthesized by reaction of III*c*–VI*c* with iodoacetyl chloride (7) or with phenyl chloroformate (9), as previously described or by suitable modification of the conditions as noted in Table I. The requisite 6-aminocoumarin-3-carboxylic acid (III*c*) was obtained by stannous chloride reduction of 6-nitrocoumarin-3-carboxylic acid (III*b*) (13); catalytic reduction gave deeply colored amorphous products that could not be purified. 6-Amino-2-hydroxycinchoninic acid (IV*c*) was best prepared by ferrous ammonium sulfate reduction of the nitro acid (IV*b*) (14); in this case catalytic reduction gave poor and unreproducible yields of IV*c*. Catalytic reduction of 4-hydroxy-6-nitroquinoline-3-carboxylic acid (V*b*) in the presence of two equivalents of hydrochloric acid afforded 6-amino-4-hydroxyquinoline-3-carboxylic acid (V*c*) in 37% yield.

Although condensation of 4-nitro-*o*-phenylenediamine (VIII) with ethyl oxomalonate might be expected to give two isomers, VII and X, only one isomer could be isolated. Since the reaction proceeds by initial condensation of the oxo-group of IX with the stronger *meta*-amino group of VIII, the product could be expected to be VII. Analogous reactions involving *o*-diamines of differing amine strengths



have been described (15, 16). Saponification of VII afforded VI*b* in 77% yield. Considerable difficulty was encountered in reducing VI*b* to the amine VI*c*. Catalytic reduction frequently led to over-reduction products. The best method found for this reduction was ferrous ammonium sulfate; the product was easily isolated in pure form, but the yields were only fair.

A candidate for an exo-alkylating inhibitor with a more reactive alkylating group was 5-(bromoacetyl)-salicylic acid (XII). This compound was prepared by bromination of 5-acetylsalicylic acid (XI) (17) in ether.



That the bromine had entered the methyl group and not the ring was shown by conversion of XII to the pyridinum betaine (XIII) and by the high reactivity of the halogen of XII towards thiosulfate (Table II). Compound XII has the same melting point described for XII prepared by another route (18) that appeared technically unattractive.

Synthesis[3]

6-Nitrocoumarin-3-carboxylic Acid (*IIIb*).—Condensation of 10 Gm. of 5-nitrosalicylaldehyde with 10 Gm. of ethyl malonate in 200 ml. of boiling ethanol containing 1 ml. of piperidine gave a 98% yield of ethyl 6-nitrocoumarin-3-carboxylate, m. p. 194–196° [lit. m.p. 198° (13)]. Considerable difficulty was experienced in finding proper conditions for saponification of the ester. Precipitation by ordinary acidification gave a product still containing potassium and apparently containing an opened lactone ring. The following procedure gave a good product each time.

A mixture of 1.00 Gm. of ester and 10.6 ml. of 5% alcoholic potassium hydroxide was stirred for 90 minutes, during which time the orange potassium salt of the product separated. Sufficient water was added to just dissolve the potassium salt; the solution was added in a thin stream with good mechanical stirring to 60 ml. of 3 *N* hydrochloric acid. The product, which gradually separated, was collected on a filter, washed with water, and recrystallized from ethyl acetate; yield, 0.50 Gm. (56%), m.p. 227–229° [lit. m.p. 234–235° (13)].

6-Aminocoumarin-3-carboxylic Acid (*IIIc*).—A 2.00-Gm. (9.5 mmole) quantity of *IIIb* was added to 6 Gm. of stannous chloride dihydrate in 6 ml. of 12 *N* hydrochloric acid. The mixture was warmed gently on the steam bath until the exothermic reaction had started, then was controlled by cooling in an ice bath. The solution was then heated on the steam bath for 2 hours. Spin-evaporation *in vacuo* gave a thick syrup to which 100 ml. of water was added. The yellow product gradually crystallized; yield, 1.8 Gm. (95%), m.p. 224–226°, that was suitable for further transformations. Recrystallization from dilute alcohol gave crystals, m.p. 234°; $\nu_{max.}^{KBr}$ 3400 (OH, NH); 1725 (lactone and carboxyl C=O); no nitro bands near 1520 and 1340 cm.$^{-1}$.

Anal.—Calcd. for $C_{10}H_7NO_4 \cdot H_2O$: C, 53.8; H, 4.07; N, 6.27. Found: C, 53.6; H, 4.01; N, 6.26.

6-Amino-2-hydroxycinchoninic Acid (*IVc*).—A solution of 2.0 Gm. (8.5 mmoles) of *IVb* (14) in 6 ml. of water and 6 ml. of concentrated ammonium hydroxide was poured with stirring into a boiling solution of 23 Gm. of ferrous ammonium sulfate hexahydrate in 50 ml. of water. Then 20 ml. of concentrated ammonium hydroxide was added in small portions over a period of 20 minutes with stirring, but without additional heating. The mixture, after being heated an additional 30 minutes on the steam bath, was filtered, and the cooled filtrate acidified to about pH 4 with acetic acid. The product was collected on a filter and washed with cold water; yield 1.0 Gm. (57%), m.p. above 300°; this material is suitable for further transformations. Recrystallization from water gave pale yellow needles, m.p. above 300°, whose infrared spectrum indicated a zwitterion structure; $\nu_{max.}^{KBr}$ 3450 (NH); 1670 (amide C=O); 1600 cm.$^{-1}$ (carboxylate). Catalytic reduction was not as satisfactory since the product could not always be isolated and at best gave a 46% yield.

Anal.—Calcd. for $C_{10}H_8N_2O_3$: C, 58.8; H, 3.93; N, 13.7. Found: C, 58.5; H, 4.24; N, 13.5.

6-Amino-4-hydroxyquinoline-3-carboxylic Acid (*Vc*).—A mixture of 1.00 Gm. (4.2 mmoles) of *Vb*, 200 ml. of 70% ethanol, 0.083 ml. of 12 *N* hydrochloric acid, and 100 mg. of platinum oxide was shaken with hydrogen at 2–3 Atm. until reduction was complete (about 24 hours). The filtered solution was spin-evaporated to dryness *in vacuo* leaving 0.20 Gm. of product. An additional 0.10 Gm. was obtained by extracting the catalyst precipitate with 5 ml. of 1 *N* NaOH, then acidification of the filtrate; total yield, 0.30 Gm. (37%), m.p. above 300°, that was suitable for the next step. Recrystallization from ethanol-water gave the analytical sample, m.p. above 300°; $\nu_{max.}^{KBr}$ 3500, 3400 (NH); 1675 (carboxyl C=O), 1620 cm.$^{-1}$ (C=N or amide C=O).

Anal.—Calcd. for $C_{10}H_8N_2O_3$: C, 58.8; H, 3.93; N, 13.7. Found: C, 58.7; H, 3.80; N, 13.6.

Ethyl 3-Hydroxy-7-nitroquinoxaline-2-carboxylate (VII).—A 6.96-Gm. (0.04 mole) quantity of ethyl ketomalonate was added to a mixture of 6.12 Gm. (0.04 mole) of 4-nitro-*o*-phenylenediamine and 125 ml. of absolute ethanol. The mixture was refluxed with gentle stirring for 7 hours. The hot solution was filtered, the filtrate was diluted with 100 ml. of water, treated with 1 Gm. of charcoal at the boiling point, then filtered. On being cooled, the filtrate deposited 5 Gm. (48%) of product, m.p. 225–227°. By recrystallization from absolute ethanol an analytical sample was obtained, m.p. 227°; $\nu_{max.}^{KBr}$ 3500 (NH); 1750 (ester C=O), 1652 (amide C=O); 1525 cm.$^{-1}$ (NO_2).

Anal.—Calcd. for $C_{11}H_9N_3O_5$: C, 50.2; H, 3.42; N, 15.9. Found: C, 50.2; H, 3.54; N, 15.7.

3-Hydroxy-7-nitroquinoxaline-2-carboxylic Acid (*VIb*).—A mixture of 3.0 Gm. (11.4 mmoles) of ethyl ester (VII) and 60 ml. of 3 *N* sodium hydroxide was stirred for 4 hours. Then 500 ml. of water was added and the nearly clear solution was filtered. The filtrate was added to 60 ml. of 3 *N* hydrochloric acid with stirring. The yellow product was collected on a filter and washed with water; yield, 1.8 Gm. (67%), m.p. 265° dec. suitable for the next step. For analysis a sample was recrystallized from 2-methoxyethanol to give yellow crystals, m.p. 267° dec.; $\nu_{max.}^{KBr}$ 3500 (NH); 1750 (carboxyl C=O); 1640 (amide C=O); 1525 cm.$^{-1}$ (NO_2).

Anal.—Calcd. for $C_9H_5N_3O_5$: C, 45.9; H, 2.14; N, 17.9. Found: C, 46.1; H, 2.31; N, 17.9.

3-Hydroxy-7-aminoquinoxaline-2-carboxylic Acid (*VIc*).—This was prepared by ferrous reduction of *VIb* as described for the *preparation of* *IVc* in 40% yield, m.p. above 300°. A sample was dissolved in 10 ml. of 1 *N* sodium hydroxide for analysis. The solution was filtered, then acidified with 3 *N* hydrochloric acid with ice cooling; the product was collected and washed with water. This reprecipitation was repeated once more to give yellow crystals, m.p. above 300°; $\nu_{max.}^{KBr}$ 3400, 3350, 3200 (NH); 1690 (carboxyl C=O); 1630, 1580 cm.$^{-1}$ (C=N or amide C=O).

[3] Melting points were taken in capillary tubes in a Meltemp block and are uncorrected. Infrared spectra were determined in KBr pellets with a Perkin-Elmer 137B recording spectrophotometer. Enzyme kinetics and ultraviolet spectra were determined with a Cary 11 recording spectrophotometer.

Anal.—Calcd. for $C_9H_7N_3O_3$: C, 52.7; H, 3.44; N, 20.5. Found: C, 52.6; H, 3.58; N, 20.2.

6-(Carbophenoxyamino)coumarin-3-carboxylic Acid (IIIe).—A 1.04-Gm. (6.72 mmoles) quantity of phenyl chloroformate in one portion was added to a rapidly stirred solution of 1.00 Gm. (4.4 mmoles) of 6-aminocoumarin 3-carboxylic acid (III*c*) in 50 ml. of water containing 1.12 Gm. of sodium bicarbonate and cooled to 3° in an ice bath. After being stirred for 3 hours in an ice bath, during which time part of the yellow product separated as the sodium salt, the mixture was filtered. The filtrate was acidified to pH 3 with 3 *N* hydrochloric acid; the product was collected on a filter and washed with water.

The insoluble sodium salt was added to 50 ml. of water preheated to 70°. After being stirred for a few minutes, the solution was cooled, acidified, and the product collected. This process should be repeated until no more product is obtained on acidification of an extract. Extended heating of the sodium salt in water causes hydrolysis of the carbophenoxy group.

The dried, combined precipitates were recrystallized from ethyl acetate to give yellow crystals; yield, 0.75 Gm. (52%), m.p. 218–220°; $\nu_{max.}^{KBr}$ 3300 (NH); 1730 (lactone C=O); 1705 (urethane C=O); 1690 (carboxyl C=O); 737 and 685 cm.$^{-1}$ (monosubstituted phenyl). (See Table I for analytical data.)

All compounds prepared by this method are listed in Table I under *Method A*. In some cases, the sodium salt was soluble and all of the product was obtained by acidification of the filtered alkaline solution (*Method B*); in some cases all of the product separated as sodium salt and none was obtained by acidification of the filtered alkaline solution (*Method C*). In one case, the sodium salt of the starting material was insoluble at 0° and it was necessary to run the reaction at 40° (*Method D*).

4-Hydroxy-6-(iodoacetamido)quinoline-3-carboxylic Acid (V*d*).—To a stirred solution of 250 mg. (1.2 mmoles) of V*c* in 5 ml. of 2% sodium hydroxide (2.4 mmoles) cooled in an ice bath was added 0.45 Gm. (2.4 mmoles) of iodoacetyl chloride in one portion. The mixture was stirred for 30 minutes, then the product (free acid) was collected on a filter and washed with water; yield, 270 mg. (61%), m.p. above 300°. Recrystallization from 2-methoxyethanol by addition of water gave the pure product, m.p. above 300°; $\nu_{max.}^{KBr}$ 3400 (NH); 1660 (amide and carboxyl C=O); 1600 cm.$^{-1}$ (C=C). (See Table I for analytical data.)

Compound V*c* could not be acylated by *Method A* in sodium bicarbonate, since the proton was not removed from the zwitterion with aqueous sodium bicarbonate. Compounds prepared by this route are listed in Table I under *Method E*.

5-(Bromoacetyl)salicylic Acid (XII).—A solution of 0.28 ml. of bromine in 50 ml. of ether over a period of 2 hours was added to a magnetically stirred solution of 0.92 Gm. (5.11 mmoles) of 5-acetylsalicylic acid (XI) (17) in 100 ml. of reagent ether protected from moisture. The filtered solution was spin-evaporated to dryness *in vacuo;* yield, 1.3 Gm. (98%), m.p. 132°. Recrystallization from chloroform gave 0.90 Gm. (68%) of white crystals, m.p. 152°; $\nu_{max.}^{KBr}$ 3100 (OH); 1670 cm.$^{-1}$ (C=O).

Anal.—Calcd. for $C_9H_7BrO_4$: C, 41.7; H, 2.70; Br, 30.7. Found: C, 41.9; H, 2.89; Br, 30.6.

The halogen was lost if the compound was recrystallized from protic solvents; the compound should be stored in a desiccator. A melting point of 152° has been reported for XII prepared by a different route (18).

5-(ω-Pyridiniumacetyl)salicylate Betaine (XIII).—A solution of 1.0 Gm. (3.8 mmoles) of XII in 10 ml. of reagent pyridine was heated on a steam bath for 30 minutes. On cooling, 1.0 Gm. of a crystalline precipitate was obtained that was a mixture of XIII and its hydrobromide salt, m.p. 180–200° dec. A hot solution of the salt in 20 ml. of water was neutralized with a solution of 0.24 Gm. of anhydrous sodium acetate dissolved in 0.5 ml. of water. After standing overnight at 3°, the mixture was filtered and the product washed with ice water; yield, 0.42 Gm. (42%), m.p. 262–264°. Recrystallization from water gave white crystals, m.p. 271°.

Anal.—Calcd. for $C_{14}H_{11}NO_4$: C, 65.4; H, 4.30; N, 5.44. Found: C, 65.1; H, 4.46; N, 5.37.

RESULTS[4]

Comparison of Halogen Reactivities.—When the rate data with sodium thiosulfate and the iodo compounds were plotted as second-order reactions, straight lines were obtained. The slope of a given line gave the relative halogen reactivity compared to 4-ISA (4, 12). The relative reactivities are recorded in Table II where 4-ISA is given an arbitrary value of 1.0. The bromoketone, XII, was 19 times as reactive as 4-ISA. The iodoacetamido derivatives (III*d*–VI*d*) had the same order of halogen activity as 4-ISA, as expected from previous comparisons (12, 19).

K_I Values of Inhibitors.—The I_{50} value[1] could be determined from a plot of Vo/V against I where Vo = velocity of the enzyme reaction without inhibitor, and V = velocity in the presence of I concentration of inhibitor. Where $Vo/V = 2$, $I = I_{50}$; the K_I could be calculated (9) from

$$K_I = K_m \times I_{50} \qquad \text{(Eq. 2)}$$

Both α-ketoglutarate and pyruvate had $K_m = 2.5 \times 10^{-4}$ in our system. These K_I values are listed in Table II.

In some cases noted in Table II, the I_{50} concentration could not be reached because of insufficient transmittance of light at 340 mμ; absorption by the compound did not allow observation of the decrease in 340 mμ absorption necessary for following the enzyme rate. In such cases, the near maximum Vo/V obtainable with the slits not quite wide open in the spectrophotometer could be used for calculating the K_I; obviously these results will have about twice the error in the absolute value of K_I at I_{20} or I_{30}. These I_{20} or I_{30} could be converted to K_I values by use of the corresponding I_{20} or I_{30} values of 4-ISA and the $K_I = 17 \times 10^{-4}$ for the latter compound. Since I_{50} measures the amount of EI complex at 50% inhibition and I_{20} measures the amount of EI complex at 20% inhibition, the ratio of Eq. 3 is derivable. Thus from the I_{20} and I_{50} values of 4-ISA and

$$I'_{20}/I_{20} = I'_{50}/I_{50} = K_I'/K_I \qquad \text{(Eq. 3)}$$

[4] The capable technical assistance of Maureen Vince and Dorothy Ackerman is gratefully acknowledged.

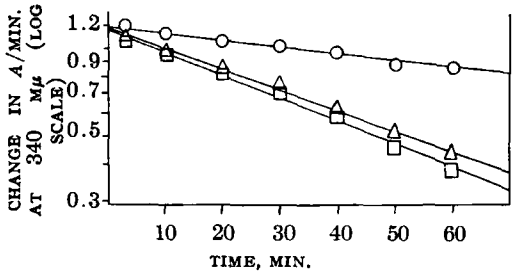


Fig. 1.—Comparative irreversible inhibition of LDH·DPNH by 4-ISA and 5-(bromoacetyl)salicylic acid (XII). Key: ○, LDH·DPNH control; △, 2 m*M* 4-ISA; □, 0.2 m*M* XII.

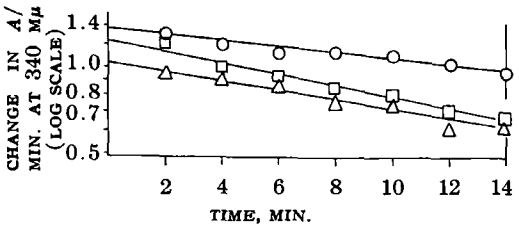


Fig. 2.—Comparative irreversible inhibition of GDH·DPNH by 4-ISA and 6-carbophenoxyamino-2-hydroxycinchoninic acid (IV*e*). Key: ○, GDH·DPNH control; △, 2 m*M* 4-ISA; □, 0.2 m*M* IV*e*

the I_{20} of an unknown compound, the K_I of an unknown compound can be estimated. The K_I values calculated from I_n values less than I_{50} are noted in Table II.

5-(Bromoacetyl)salicylic acid (XII) was reversibly bound to LDH and GDH slightly stronger than 4-ISA. The carbophenoxyamino (III*e*–V*e*) and iodoacetamido (III*d*–VI*d*) heterocyclic carboxylic acids were bound to LDH and GDH 3.5 to 15 times stronger than 4-ISA.

Irreversible Inhibition by Compounds in Table II.—The inactivation rates of LDH and GDH by 4-ISA are assigned arbitrary values of 1.0; it should be noted that the absolute rates of inactivation of the two enzymes by 4-ISA are not identical. The relative rate of inactivation by a given compound is listed in Table II. In each experiment, 4-ISA was compared simultaneously with the new compound, since for reasons still unknown (4) the absolute rate of inactivation of the enzyme can vary twofold from experiment to experiment, but the relative rates usually duplicate with 30%.

The halogen of 5-(bromoacetyl)salicylic acid (XII) was 19 times as reactive as that of 4-ISA, and XII irreversibly inhibited LDH at 120% rate of 4-ISA with concentrations of 0.2 m*M* and 2 m*M*, respectively (Fig. 1); 0.2 m*M* of XII also irreversibly inhibited GDH at 65% of rate of 4-ISA at 2 m*M*.

None of the carbophenoxyamino compounds (III*e*–V*e*) irreversibly inhibited LDH, but two irreversibly inhibited GDH at one-tenth the concentration needed for 4-ISA. For example, 6-carbophenoxyamino-2-hydroxycinchoninic acid (IV*e*) at 0.2 m*M* give about the same rate of inactivation of GDH as 2 m*M* 4-ISA (Fig. 2); IV*e* also showed a rate saturation effect (Fig. 3).

Three of the four iodoacetamido compounds (III*d*, IV*d*, VI*d*) at one-tenth to one-fifth the concentration of 4-ISA showed irreversible inhibition of LDH at 45–83% the rate shown by 4-ISA. An example is 7 - (iodoacetamido) - 3 - hydroxyquinoxaline - 2-carboxylic acid (VI*d*) shown in Fig. 4. The fourth compound (V*d*) was a strong reversible inhibitor of LDH but showed no irreversible inhibition.

DISCUSSION

As shown in Fig. 1 and Table II, 0.2 m*M* of 5-(bromoacetyl)salicylic acid (XII) irreversibly inhibited LDH at 1.2 times the rate of 2 m*M* 4-ISA. There are four factors that can influence the rate of irreversible inhibition by exo-alkylation: (*a*) the extent of *EI* complex formation which can be calculated (4) from the K_I by Eq. 4, (*b*) the ability of the reversibly bound inhibitor to bridge to a nucleophilic group on the enzyme surface adjacent to the active site (12, 19), (*c*) the nucleophilic character of the

$$EI = \frac{E_t}{\frac{K_I}{I} + 1}$$

where E_t = total enzyme concentration (Eq. 4)

enzyme being alkylated (9, 11), and (*d*) the electrophilic character of the alkylating group. If factors (*b*), (*c*), and (*d*) were identical for 4-ISA and XII, then XII should have irreversibly inhibited LDH at about 3.8 times the rate of 4-ISA, which can be calculated from the ratio = 19 × 0.13/0.70, where 19 is the increased thiosulfate reactivity, 0.70 is the fractional amount of enzyme in the *EI* complex with 2 m*M* 4-ISA, and 0.13 is the fractional amount of enzyme in *EI* complex with 0.2 m*M* of XII. Therefore, factors (*b*), (*c*), or (*d*) or any combination of the three are contributing to the ratio of irreversible inhibition. In addition, the enzymic nucleophilic group, if not SH, probably has a different ratio of rate of reaction between XII and 4-ISA than the 19 observed with thiosulfate.

It can be calculated from Eq. 4 that at a concentration of 0.2 m*M* only 13% of total LDH is reversibly complexed by XII. This 13% appears to be close to the point of bimolecular alkylation of other enzymes where no complexing is required, which could be quite disadvantageous in the intact cell. In fact, however, the practically nonexistent energy barrier for complexing contrasts sharply with the energy barrier involved in a bimolecular reaction; this differential has been given an elegant mathematical treatment by Singer, *et al.* (5).

Although the carbophenoxy compounds in Table II were strongly bound to LDH reversibly, these showed no irreversible inhibition; the lack of irreversible inhibition of LDH by other carbophenoxy compounds has been previously noted (9, 11). Since all but one of these earlier carbophenoxy compounds could irreversibly inhibit GDH (11), it was not surprising that the carbophenoxy compounds, III*e*–IV*e*, irreversibly inhibited GDH. Of importance to this discussion is the low concentrations necessary for irreversible inhibition, as expected from the low K_I values; only a concentration one-

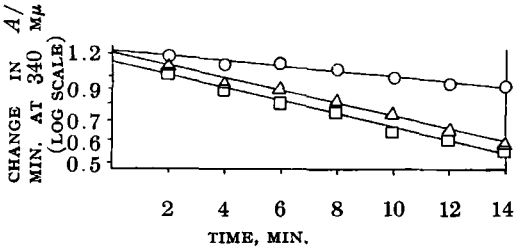


Fig. 3.—Rate saturation of irreversible inhibition of GDH·DPNH by 6-carbophenoxyamino-2-hydroxycinchoninic acid (IV*e*). Key: ○, LDH·DPNH control; △, 0.15 m*M* of IV*e*; □, 0.30 m*M* of IV*e*.

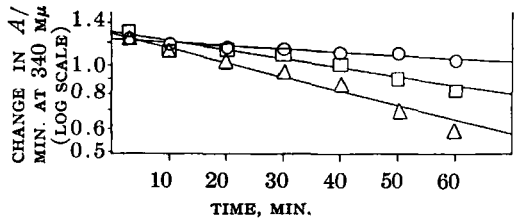


Fig. 4.—Comparative irreversible inhibition of LDH·DPNH by 4-ISA and 7-(iodoacetamido)-3-hydroxyquinoxaline-2-carboxylic acid (VI*d*). Key: ○, LDH·DPNH control; △, 2 m*M* 4-ISA; □, 0.2 m*M* VI*d*.

tenth that of 4-ISA (Table II) or one-twentieth of other carbophenoxyamino derivatives of salicylic acid (11) was required. As yet no carbophenoxy compounds have been found that give irreversible inhibition of LDH by the exo-alkylation mechanism, but the search for such a compound is continuing.

One of the carbophenoxy compounds was investigated further to demonstrate that reversible complexing at the active site was a necessary prerequisite for irreversible inhibition. 6-Carbophenoxyamino-2-hydroxycinchoninic acid (IV*e*) showed a rate saturation effect (Fig. 3); doubling the concentration of inhibitor from 0.15 m*M* to 0.30 m*M* gave an increase in inactivation rate of 1.1, somewhat less than the calculated ratio of 1.4 from Eq. 4 and definitely less than 2.0 if the inactivation occurred by bimolecular reaction (tail-alkylation). In a check run comparing 0.12 m*M* and 0.24 m*M*, the ratio of inactivation rates was 1.4 compared to a calculated value of 1.5.

All of the iodoacetamido compounds (III*e*–VI*d*) showed reversible inhibition of LDH with K_I's one-fifth to one-twelfth of 4-ISA. In addition, three of the four showed at least one-half the rate of irreversible inhibition of 4-ISA at one-fifth to one-tenth the concentration required for 4-ISA. The fourth compound (V*d*) showed no irreversible inhibition. This difference is most probably because of the bridge principle of specificity (12, 19), since in V*d* the distance of the iodomethylene group from the 1,2-oxo-carboxylic acid groups involved in binding is quite different than the distance between the corresponding groups of III*d*, IV*d*, and VI*d*.

The fact that a number of these compounds with lower K_I could inhibit LDH and GDH at correspondingly lower concentrations than used for 4-ISA gives another line of evidence supporting the exo-alkylation phenomenon as an explanation for the mode of selective denaturation of LDH and GDH by these alkylating inhibitors.

It can be concluded that in the design of an exo-alkylating irreversible inhibitor for a given enzyme careful attention should be given to use compounds that give the most tight-reversible binding possible. The more reactive bromoacetyl group of XII gives faster inactivation and allows design of a potential *in vivo* inhibitor that can vary in alkylating ability from the slow-reacting chloroacetamido group to the fast-reacting iodomethyl ketone group; in this way the proper balance between the highest reactivity with the least destruction of inhibitor by random reactions prior to the attack of the target site by the irreversible inhibitor can be maintained.

By placing the bromoacetyl group on III–VI, compounds should be obtained that would give irreversible inhibition of LDH *in vitro* at concentrations approaching 10^{-5} *M*, within the utilizable range for *in vivo* activity. As pointed out earlier (1, 4), our *in vitro* approach must by necessity ignore for the time the important problems of transport and membrane permeability present in an *in vivo* system. However, if necessary the carboxyl group of inhibitors such as II–VI could be masked by ester or amide functions to give a latent activity that could be regenerated by intracellular nonspecific amidases or esterases. It is our opinion that such an approach to LDH inhibitors is worthy of further pursuit.

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Peppermint and Spearmint Tissue Culture II

Dual-Carboy Culture of Spearmint Tissues

By CHING-JU WANG† and E. JOHN STABA

A 5-day old, 15 per cent (v/v) spearmint cell inoculum in a dual-carboy system containing modified T-medium with 2,4-dichlorophenoxyacetic acid (0.5 p.p.m.), Dow Corning-B antifoam (500 p.p.m.), and bacitracin (5 p.p.m.) is suggested for good cell suspension growth.

APPLICATIONS of plant tissue cultures may be forthcoming for evaluating herbicides and plant growth regulators, for the production of food, for testing drug efficacy and toxicity, and for the production of economically useful compounds. Basic microbial techniques have been used to grow large quantities of plant tissue in suspension cultures (1–3).

Air has been supplied to plant suspension cultures containing less than 0.5 L. of medium by agitating glass flasks on various shakers (4–7), or to larger volumes of medium by forcing sterile air through open-end tubing or spargers in a carboy or pilot-plant fermentor (1, 3, 8, 9). Agitation may be produced by the aeration process itself (1, 2) or by magnetic bar stirring devices (9).

This study reports the growth characteristics of spearmint (*Mentha spicata* L.) cell inocula of known cell number in dual-carboys receiving constant air flow and agitation. The effect of certain antifoam and antibiotic compounds on spearmint tissue growth is also reported.

EXPERIMENTAL

The spearmint stem-callus tissues studied were approximately 1-year-old and had been established by the method previously published (7). Suspension cultures in this study were maintained and subcultured on our modification of Murashige's and Skoog's tobacco medium (T-medium) with 0.5 p.p.m. of 2,4-dichlorophenoxyacetic acid (2,4-D). Murashige's and Skoog's tobacco medium (10) was referred to as modified Murashige's medium in our previous publications (7, 11).

Erlenmeyer Flask Cultures.—Preliminary experiments were performed in 250-ml. Erlenmeyer flasks containing 50 ml. of medium to determine the effects of cell inoculum concentrations, antifoams, and antibiotics on the growth of spearmint suspension cultures.

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Portion of a thesis submitted by C. J. Wang to the Graduate College, University of Nebraska, in partial fulfillment of Master of Science degree requirements.
Presented to the Pharmaceutical Science Section, American Association for the Advancement of Science, Philadelphia meeting, December 1962.
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The 15-day-old cell inocula for these experiments were prepared by a method previously published (12). After inoculation, the flasks were incubated on a reciprocal shaker (stroke: 9 cm.; rate/minute: 88), at room temperature (about 27°) and light. Relative growth was calculated as:

$$\text{Relative Growth (\%)} = \frac{\text{Growth average (four flasks) of each experiment}}{\text{Growth average (four flasks) of control}} \times 100$$

Dual-carboy Cultures.—Each carboy of the dual-carboy system (Fig. 1) was simultaneously aerated by a forced flow of sterile, moistened air through a T tube attached to a stainless steel sparger (porosity:

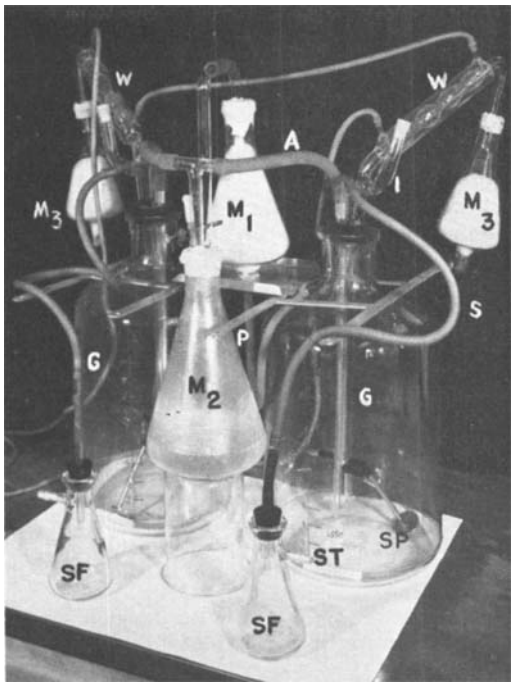

Fig. 1.—Dual-carboy apparatus. *A*, air inlet tubing connected to stainless steel tube and sparger; *I*, innoculation tube; *G*, 12-L. carboy; *M*, modified 1-L. flask with cotton for air sterilization; *M*2, 2-L. flask with distilled water for water saturation of air; *M*3, modified 500-ml. flask with cotton as air vent; *P*, air inlet tube connected to a gas flowmeter; *S*, sampling tube; *SF*, sampling flask; *SP*, stainless steel sparger; *ST*, magnetic bar; *W*, water condenser.

TABLE I.—OXYGEN ABSORPTION RATES (OAR) OF ERLENMEYER FLASKS AND CARBOYS I AND II

Aeration Condition	Volume Sulfite Soln., L.	DC-B Antifoam, p.p.m.	OAR (m*M* O_2/L./min.) Erlenmeyer[a]	Carboy I[b]	Carboy II[b]
Reciprocating shaker[c]	0.05	...	0.235	...	...
I[d]	3.0	500	...	0.068	0.066
	6.0	500	...	0.026	0.024
II[e]	3.0	500	...	0.117	0.117
	3.0	...	...	0.181	0.158
	6.0	500	...	0.058	0.055

[a] Erlenmeyer flask (250 ml.) with cotton plug. [b] Twelve-liter capacity. [c] Stroke: 9 cm.; rate/min.: 88, at room temperature. [d] Air flow: 4.5 L./min. at 28°. [e] Air flow: 27 L./min. at 28°.

TABLE II.—EFFECT OF INOCULUM SIZE ON THE GROWTH OF SPEARMINT TISSUE IN ERLENMEYER FLASKS[a]

	Inoculum Size[b] 0.25	1.0	2.0	3.0
Days	Growth[c]	Growth[c]	Growth[c]	Growth[c]
15	22.6	211.6	361.6	474.4
30	480.8	495.6	477.3	474.0

[a] Flasks (250 ml.) with 50 ml. of medium. [b] Milligrams cellular dry weight per flask. [c] Milligrams cellular dry weight after 15 or 30 days culture (average of four replicates).

TABLE III.—EFFECTS OF ANTIFOAM AGENTS ON SPEARMINT SUSPENSION GROWTH

Antifoam	Source	Compn.	Concn. Studied[a] Low	High	Relative Growth, %[b] Low Concn.	High Concn.
DC-A	Dow Corning (Mich.)	Silicone 100%	10	100	94.3	99.4
DC-B	Dow Corning (Mich.)	Silicone 10%	100	1000	99.3	96.5
FD-62	Hodag (Ill.)	Silicone 10%	100	1000	99.5	95.1
GE-60	General Electric (N. Y.)	Silicone 30%	30	300	97.7	98.2
Troy-333	Troy (N. J.)	Non-silicone 100%	10	100	104.5	99.1
Safflower oil	.....	Fatty acids	100	..	109.7	..

[a] Expressed in p.p.m. [b] Relative growth was determined after 21 days of culture. Each flask received 1 ml. of inoculum containing 1.9×10^6 cells (3.5 mg. cellular dry weight).

10 μ).[1] Agitation was produced by a 7.5-cm. Teflon-coated magnetic bar rotating at 90 r.p.m. on the base of each carboy. Two rates of air flow were studied; 4.5 L./minute (aeration condition I) and 27 L./minute (aeration condition II). The air flow was measured through a gas-flow meter[2] at room temperature. Pressures over the sulfite medium were 1.16 Atm. for the slower flow rate and 1.33 Atm. for the faster one. The dual-carboy system was mounted in a water bath maintained at 28°. Measured temperature variance of the medium within the carboys was negligible.

Oxygen absorption rates (OAR) of aqueous sulfite solutions in the dual-carboy system and Erlenmeyer flasks under the conditions specified in Table I were determined by standard procedures (13, 14).

The inocula for the dual-carboy system were prepared as follows. Approximately 100 ml. of 15-day-old cell suspension was transferred aseptically into a 2-L. Erlenmeyer flask containing 300 ml. of medium and grown for 5 days on the reciprocal shaker. This new cell suspension was then aseptically passed through a 602μ-mesh nylon sieve[3] and used as inocula for the carboys. Each carboy was inoculated with 15% by volume of new cell suspension and cultured in the water bath at 28° for 8 days. A sample was taken daily from each carboy for 8 days and examined for cell concentration, cellular dry weight, and the pH change in the medium.

Semiquantitative determinations were made on each medium sample for dextrose, fructose, and sucrose by a chromatographic procedure previously published (12). Aqueous standard solutions of sucrose (1.5%), fructose (1.5%), and dextrose (1.0%) were used. Whatman No. 1 chromatographic papers were spotted with the standard solutions (2, 4 and 10 μl.), control nutrient medium (10 μl.), and the medium filtrates (10 μl.). The presence of dextrose was confirmed by Clinistix[4] test paper.

RESULTS

Erlenmeyer Flask Cultures.—The heaviest inoculum (3 mg. cellular dry weight/flask) produced a maximum yield within 15 days (Table II). With smaller inocula (0.25, 1 and 2-mg. cellular dry weight/flask), proportionately less growth resulted within 15 days, but the cultures achieved a maximum growth within 30 days.

None of the six antifoam compounds studied at the low or high concentration levels appreciably affected spearmint tissue growth (Table III).

Amphotericin, griseofulvin, and/or oxytetracycline (5 p.p.m.) inhibited spearmint tissue suspension growth. The effects of penicillin G, bacitracin, tylosin, tylosin with nystatin, and nystatin are shown in Table IV. Tylosin and griseofulvin were autoclaved with the medium, but the other antibiotics were added aseptically to the medium after dilution with sterilized double-distilled water.

[1] Scientific Glass Apparatus Co., Inc., Bloomfield, N. J.
[2] Brooks Rotameter Co., Lansdale, Pa.
[3] Trobler, Ernst and Traber, Inc., New York, N.Y.
[4] Ames Co., Inc., Elkhart, Ind.

TABLE IV.—EFFECT OF ANTIBIOTICS ON SPEARMINT SUSPENSION GROWTH

Antibiotic	Concn.[a]	Relative Growth, %[b] Generation 1	Generation 2	Generation 3
Penicillin G[c]	60	101.7	...	...
Bacitracin[c]	5	92.8	106.3	...
Tylosin[c]	100	46.9	112.3	100.7
Nystatin and tylosin[c]	25 20	23	...	...
Nystatin[d]	25	52.2	...	...
	50	11.8	...	...
	125	0.0	...	...

[a] Expressed in p.p.m., except nystatin which is expressed as u./ml. [b] Determined after 15 days of culture. [c] Each flask received 4 ml. of inoculum containing 872 × 10^3 cells (2.3 mg. cellular dry weight). [d] Each flask received 4 ml. of inoculum containing 338 × 10^3 cells (1.6 mg. cellular dry weight).

Bacitracin (5 p.p.m.) was routinely used as a constituent in the medium for dual-carboy cultures.

The OAR values obtained under certain specified conditions for 250-ml. Erlenmeyer flasks and the dual-carboy system are shown in Table I. At no time did the OAR values for the sulfite medium in the dual-carboy system exceed the value for the flasks.

Dual-carboy Cultures.—Preliminary growth studies in the dual-carboy system indicated that a 15% (v/v) inoculum resulted in greater yields than a 5% (v/v) inoculum, and that a 5-day-old inoculum resulted in greater yields than did a 30-day-old inoculum.

The results obtained from carboy I under aeration condition I are shown in Fig. 2. The growth yield of spearmint tissue in carboy I under aeration condition I was 112.13 Gm. fresh weight, and that in carboy I under aeration condition II 95.70 Gm. fresh weight after an 8-day growth cycle. The growth yield in carboy II was 10% of that of carboy I under aeration condition I and 40% of carboy I under aeration condition II.

The utilization of sucrose, fructose, and dextrose by tissues in carboy I grown under aeration condition I is shown in Fig. 3.

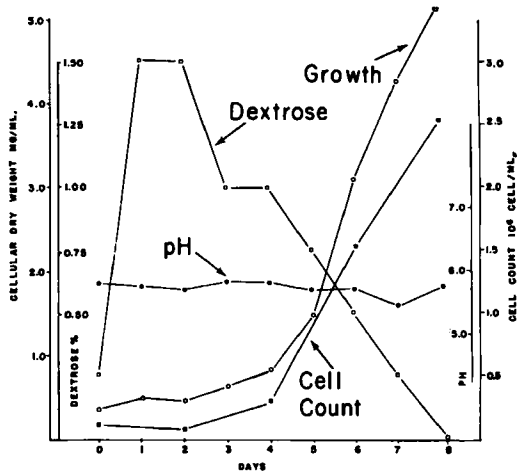

Fig. 2.—Spearmint suspension growth in carboy I containing 3 L. of modified T-medium with DC-B antifoam (500 p.p.m.) and bacitracin (5 p.p.m.). Inoculum: age, 5 days; quantity, 15% v/v; dry weight, 2 mg./ml.; cell number, 824 × 10^3 cells/ml. Final growth yield under aeration condition I: 112.13 Gm. fresh weight.

DISCUSSION

Torrey (15) has suggested that the smaller the inoculum the more complex the medium must be to reduce the time required for cells to "condition" the medium. Blakely and Steward (16) have reported that filtered and rigorously washed low-cell-content inocula of angiosperm cells on agar medium exhibited a very low frequency of colony formation. Our results indicate that the smaller the inoculum concentration of rigorously washed spearmint cells, the longer the lag phase of suspension growth. After 30 days of culture, however, the four inoculum concentrations tested resulted in a similar growth yield (Table II).

Tulecke and Nickell (2) used 300 p.p.m. of Dow Corning-A (DC-A) in their carboy cultures of various plant tissues with no apparent toxicity. We selected DC-B (500 p.p.m.) for routine use because of its lack of toxicity to spearmint suspension culture and the ease with which it may be dispersed in an aqueous medium.

Certain antibiotics have been reported to control microbial contamination in plant tissue cultures (2, 3, 17–19). However, none appear to have been accepted as a routine constituent of plant-growth media. Although nystatin (25 u./ml.) with tylosin (20 p.p.m.) is reported not to inhibit carrot, grape, and rose suspension cultures, we found that nystatin (25 u./ml.) or nystatin (25 u./ml.) with tylosin (20 p.p.m.) will inhibit spearmint suspension growth. Penicillin (10 u./ml.) and bacitracin (5 p.p.m.) is reported to stimulate the growth of sorrel tumor tissue (17), and penicillin (20 p.p.m.) not to inhibit Ginkgo-pollen, holly-stem, or rose-stem-callus suspension growth (2). Penicillin (60 p.p.m.) and bacitracin (5 p.p.m.) were not inhibitory to spearmint suspension cultures. Bacitracin (5 p.p.m.) was routinely used in media for dual-carboy cultures.

Convolvulus cell suspensions are reported to divide most frequently 1 week after inoculation and *Haplopappus* cell suspensions 3 days after inoculation (20). Similarly, our preliminary studies indicated that a 5-day-old, 15% v/v inoculum passed through a 602 μ-mesh nylon sieve gave more growth in the dual-carboy system than either 15 or 30-day-old tissue inocula.

Plant tissue culture growth may be reported as Growth Index (GI) values

$$GI = \frac{\text{Final fresh weight growth}}{\text{Initial fresh weight inoculum}} \quad (2).$$

The Growth Efficiency Index (GEI) value suggested

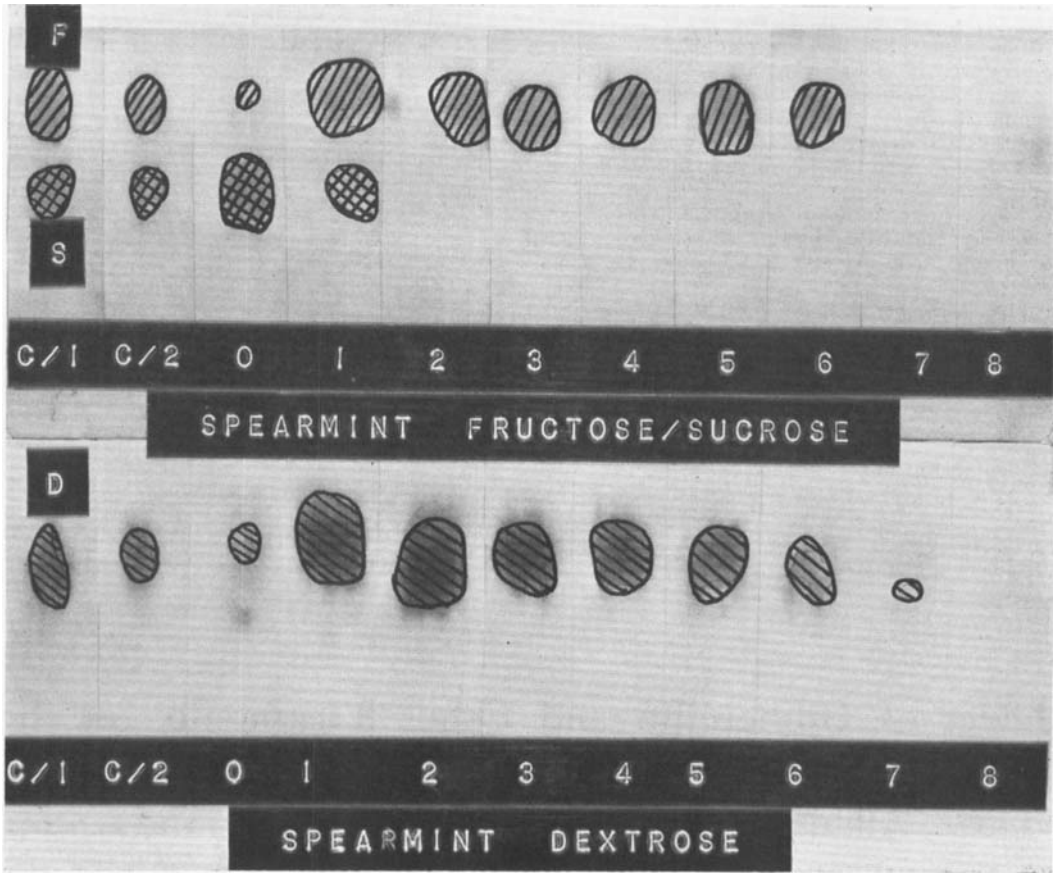

Fig. 3.—Chromatograms of fructose (*F*), sucrose (*S*) and dextrose (*D*) samples (10 μl.) taken at daily intervals (0–8) from the spearmint suspension culture described in Fig. 2. C/1: control *F*, *S*, *D*, (0.15 mcg.); C/2: control *F*, *S*, *D*, (0.075 mcg.).

below would consider the GI at maximal growth, as well as the volume of medium used and the time required for maximal tissue growth. The GEI is expressed as

$$\text{GEI} = \frac{\text{GI at maximal growth}}{\text{Medium (L.)} \times \text{days required for maximum growth}}$$

The GI and GEI values calculated for Ginkgo-carboy cultures were 8.7 and 0.03, respectively (2), and 14.9 and 0.11 for rose carboy cultures, respectively (1). The GI and GEI values for the spearmint carboy culture shown in Fig. 2 are 20.8 (112.13/5.4) and 0.87 (20.8/3 x 8), respectively. The GI and GEI values shown in Table II for the 3.0-mg. dry weight inocula grown in 250-ml. Erlenmeyer flasks are 157.3 (3.46/0.22) and 209.7 (157.3/0.05 × 15), respectively. Dry weight of spearmint cell tissues may be converted to approximate tissue fresh weight by the factor 7.3.

The poor growth repeatedly obtained in carboy II of the dual-carboy system suggested that the stainless steel sparger used in carboy II might be toxic to spearmint suspension cultures. Dyer and Richardson (21) reported that types 316 and 304 stainless steels occasionally slightly inhibited algal cultures. The effects of the presence of six different stainless steel spargers on spearmint suspension growth were tested by growing each sparger separately with cells in Erlenmeyer flasks. Various degrees of growth inhibition were observed, varying from complete inhibition or good growth with blackening of the suspension medium, to normal growth.

Spearmint suspensions grown in carboy I gave similar growth yields with either 4.5 L. of air/minute or 27 L./minute. Therefore, it appears that the amount of air supplied by the lower flow rate is adequate for good growth of spearmint tissue in the dual-carboy system used.

Sequoia semipervirens callus cultures and *Vinca rosea* crown-gall callus cultures hydrolyze certain oligosaccharides (22). Tulecke, *et al.* (23), reported that fructose and dextrose predominated in Ginkgo pollen itself. Bürstrom (24) demonstrated that excised roots hydrolyze sucrose in liquid media. In carboy I of the dual-carboy system (Figs. 2 and 3) sucrose was completely hydrolyzed to dextrose and fructose by the third day of culture. Both dextrose and fructose were absent in the culture medium after 8 days.

CONCLUSIONS

A dual-carboy system enabled 112.13 Gm. of spearmint tissue (GI, 20.8; GEI, 0.87) to grow in one carboy receiving an inoculum of known cell volume (15% v/v), age (5 days), cell number

(824×10^3 cells/ml.), and air flow (4.5 L. air/minute) within 8 days. An air flow rate of 27 L./minute did not increase growth materially.

Spearmint suspensions in the dual-carboy system completely hydrolyzed sucrose to dextrose and fructose by the third day of culture. Both dextrose and fructose were absent in the culture medium after 8 days.

None of six antifoam compounds studied appreciably affected spearmint suspensions. Dow Corning-B (500 p.p.m.) is suggested for foam control.

Five parts per million of either amphotericin, griseofulvin, and/or oxytetracycline inhibited spearmint suspension growth. Bacitracin (5 p.p.m.) is suggested for bacterial prophylaxis.

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Effect of Gibberellin and Other Treatments on the Germination and Subsequent Biogenesis of Alkaloids in *Datura stramonium* Linné

By EDWARD L. CALDWELL† and LEO A. SCIUCHETTI

Stramonium **seeds were subjected to two "cold treatments," to treatment with gibberellic acid (GA) and with concentrated sulfuric acid (SA). The rate and percentage of germination were markedly reduced by the cold treatments, whereas they were significantly increased by the GA and SA treatments. A threefold increase in germination percentage was noted in the former group and a twofold increase in the latter. Plants from the GA, SA, and control groups were allowed to grow to maturity. Characteristic gibberellin effects were not noted in plants receiving a gibberellin-seed treatment. However, the early growth of this group was markedly reduced. At the final harvest total plant weight was slightly less than controls. Fluctuations in the alkaloid concentration of the plant organs were noted. At the final harvest the total content of alkaloids was about 87 per cent of controls. The growth rate of the SA group was considerably lower than controls initially, but, the total dry weight at the final harvest approximated the controls. Both decreases and increases in the concentration of alkaloids in the plant organs were observed. The total plant alkaloids of this group at the final harvest were about the same as controls.**

Treatment of seeds with gibberellic acid (GA) has hastened germination and promoted an earlier and more uniform emergence of the seedlings of many crop plants (1–8) and several medicinal plants (9–11). Treatment of belladonna seeds with concentrated sulfuric acid resulted in an increase in the germination rate (12). Acid scarification and various types of "cold treatments" have been recommended as pregermination treatments of seeds to stimulate germination (13, 14). A review of the literature, however, has indicated that a study of such treatments on the subsequent growth and alkaloid biogenesis of stramonium has not been conducted.

The purpose of this study was (*a*) to determine and compare the effects on the germination of stramonium seeds of a freeze-thaw method (FT), a constant freeze method (CF), treatment with GA, and treatment with concentrated sulfuric acid

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† Recipient of Kilmer Prize, 1960. Present address: Providence Hospital, Portland, Oreg.

(824×10^3 cells/ml.), and air flow (4.5 L. air/minute) within 8 days. An air flow rate of 27 L./minute did not increase growth materially.

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(SA); and (*b*) to ascertain what changes such treatment would have on the subsequent growth and alkaloid biogenesis of the plant.

EXPERIMENTAL

Procedure—Five-hundred nearly uniform seeds were selected from a 1958 seed crop of *Datura stramonium*, var. *inermis* that had been gathered in March 1959 from a plant which had been allowed to over-winter. The seeds were divided into five equal groups. Group A was treated by alternate freezing and thawing during 1 week, *i.e.*, a 24-hour freezing period was followed by a similar thawing period. Group B was kept frozen for 1 week. Group C was treated with a 50 p.p.m. solution of GA[1] for 18 hours. Group D was treated for 1 minute with concentrated sulfuric acid and then washed free of the acid. Group E received no treatment and served as controls. The seeds were dusted with Fermocide[2] and planted on April 2, 1959, under greenhouse conditions in flats containing soil composed of sand, sandy loam, and peat moss. About 50 Gm. of complete organic fertilizer[3] was thoroughly mixed into each flat of soil prior to planting. Daily counts were made for a 5-week period, even though germination had apparently ceased by the end of the third week.

Since seeds of the control group (Group E) were not soaked in distilled water for 18 hours, but were planted directly in the soil in the dry state, a subsequent experiment was performed in 1963 to determine whether the beneficial response from the treatment with the GA solution was due to in i-bition of water or to a stimulating effect of the growth regulator. One-hundred seeds were soaked for 18 hours in a 50 p.p.m. solution of GA, and a similar number was soaked for the same period of time in distilled water. The time of planting, the experimental conditions, and observation period approximated that of the initial experiment.

On May 2, the second phase of this experiment was started. Twenty-four seedlings, each from the GA, SA, and control groups were transplanted into pots containing a mixture of sand, sandy loam, peat moss, and 50 Gm. of complete organic fertilizer. Since the plants grown from seeds receiving the cold treatments did not show a significant beneficial response during germination, they were discarded.

The plants from the three groups were arranged at random in the greenhouse. On May 19 (zero time), height measurements were taken and the first harvest of plants from each of the three groups was made. Height measurements were taken twice weekly thereafter, and the remaining plants were harvested in groups of eight from each of the test groups at the end of 2 and 4 weeks. The division of the plant into its morphological parts during each harvest, fresh and dry weight determinations, pulverization, pooling, and storage of the powdered material were conducted in a manner described in a previous publication (15). The pooled samples were analyzed for total alkaloids (calculated as scopolamine) by the Brummett-Sciuchetti method (16).

Germination Observations.—The seeds first germinated on the ninth day following planting. The seeds treated with GA germinated more rapidly, and a greater per cent of them germinated than did those from the other groups (Fig. 1). The seedlings from this group appeared healthy; they did not appear chlorotic; damping-off did not occur; and satisfactory growth was sustained throughout the 5-week germination period. About a threefold increase was noted in the germination percentage of the seeds treated with GA compared with untreated seeds. About twice as many plants germinated from seeds treated with sulfuric acid as from untreated seeds. The habit of this group approximated the controls. The FT and CF groups demonstrated lower rates and percentages of germination than the controls (Fig. 1). These seedlings, likewise, appeared to be similar in appearance to the controls.

The subsequent experiment which was performed to determine whether the favorable response from soaking seeds in a gibberellin solution was due to water imbibition or to a stimulus to germination by the GA solution, demonstrated a similar, although a less pronounced trend, as that described above. For example, the number of seedlings emerging from the GA-treated group was eight at the eighth day following planting, 18 at the 12th day, 25 at the 16th day, 35 at the 20th day, 40 at the 24th day, and 40 at the 28th day. The corresponding figures for the seeds soaked in distilled water only were 1, 7, 12, 15, 16, and 16. These results indicate that the increased rate of germination and higher percentage germination was due to the stimulating effect of the GA.

Growth Observations.—The plants were observed daily during the 4-week growth period following the first harvest. There were no appreciable differences

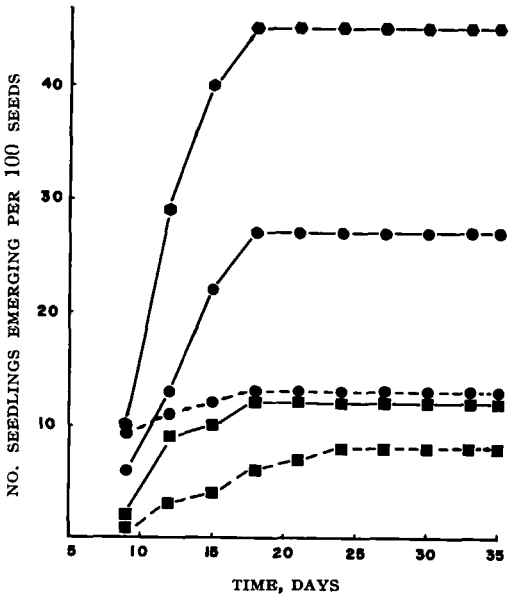

Fig. 1.—Effect of seed treatments on germination of *Datura*. Key: ●—●, gibberellic acid; ●—●, sulfuric acid; ● - - ●, controls; ■——■, freeze-thaw; ■ - - ■, constant freeze.

[1] The GA employed in this study was furnished through the courtesy of Dr. Edwin F. Alder, Agricultural Research Center, Eli Lilly and Co., Greenfield, Ind.

[2] Miller's Fermocide, 68% Ferbam, Miller Products Co., Portland, Oreg.

[3] Organic Morcrop, Chas. Lilly Co., Seattle, Wash. *Anal.*—5% total nitrogen, 3% available phosphate, 2% available potash.

TABLE I.—AVERAGE HEIGHTS[a] OF STRAMONIUM PLANTS

Treatment	Harvest Time, Wks.	Av. Height, cm.	Control, %
Control	0	11.04	...
SA	0	10.35	93
GA	0	11.16	101
Control	2	28.59	...
SA	2	28.63	100
GA	2	26.61	93
Control	4	48.63	...
SA	4	50.95	104
GA	4	44.36	91

[a] Heights in cm. are the average of eight plants.

in the habit of the treated and untreated groups. All plants appeared healthy and vigorous and none were chlorotic. Only slight changes in height were noted in the treated groups (Table I). At the final harvest, the SA group was slightly taller than controls, while the GA group was shorter. Marked stem elongation was not observed in the latter.

Fresh and Dry Weights.—Appreciable reductions in the dry weights of plants grown from treated seeds were generally noted at the first two harvests (Table II). At the terminal harvest the total dry weight of the SA group was about 96% of the controls, and that for the GA group was about 91%. These decreases were not considered significant. The growth rate of the SA group, although less than controls, was generally somewhat higher than the GA group. A study of the dry weight data indicated that the seed treatments retarded growth initially, but these plants approached the controls by the final harvest.

Concentration of Alkaloids.—The alkaloid analysis was performed on pooled samples by the Brummett-Sciuchetti method (16). Considerable fluctuations were noted in the concentration of alkaloids in the organs of the plants grown from treated seeds (Table III). Generally, slightly reduced concentrations compared with controls were noted in the leaves-tops of the treated groups. The stems of the plants grown from seed treated with sulfuric acid had an appreciably lower concentration of alkaloids at the first harvest, but a slight increase at the final harvest. A reverse trend was noted in the GA group. The roots of the SA group had a significant increase (147% of controls) at the second- and fourth-week harvests. On the other hand, the roots of plants grown from seeds treated with GA had a significant reduction at zero time but only a slight decrease at the fourth week. The SA treatment of seeds elicited a favorable response more often than did the GA treatment.

Total Plant Alkaloids.—The total alkaloids per plant and per plant organ were obtained by multiplying the dry weight of the plant part by the per cent of alkaloids obtained from the alkaloid analyses and expressing the results in milligrams (Table IV). It was noted that at each harvest plants grown from treated seeds produced less total alkaloids than the controls. The most significant decrease was at the second harvest where the total alkaloid content per plant of the GA group was only 64% of the controls. This was because of a significant decrease in plant growth and a marked reduction in the concentration of leaves-tops alkaloid. By the final harvest this group had 87% of the total alkaloids of the controls. On the other hand, the total alkaloids per plant of the SA group showed an appreciable decrease compared with controls at the first harvest, a considerable reduction at the second harvest, and approximately the same content at the final harvest. In general, the aerial parts of the treated groups showed significant decreases in total alkaloids at the first harvest, marked reductions at the second harvest, and only minor decreases

TABLE II.—WEIGHTS OF STRAMONIUM PLANT PARTS (AV./PLANT/GROUP)

Treatment and Harvest Time, Wks.	Total Wt.			Leaves-Tops			Stems			Roots		
	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %
Control, 0	3.44	0.42	...	1.71	0.23	...	0.89	0.10	...	0.84	0.09	..
SA, 0	3.53	0.37	88	1.61	0.19	83	0.80	0.07	70	1.12	0.11	122
GA, 0	3.02	0.29	69	1.57	0.16	70	0.52	0.06	60	0.93	0.07	78
Control, 2	36.58	3.49	...	22.77	2.39	...	10.50	0.78	...	3.31	0.32	...
SA, 2	29.06	2.68	76	17.61	1.86	78	8.48	0.56	72	2.97	0.26	81
GA, 2	27.09	2.60	74	16.17	1.77	74	7.31	0.56	72	3.07	0.27	84
Control, 4	82.15	11.69	...	49.12	6.80	...	23.00	3.62	...	10.03	1.27	...
SA, 4	79.90	11.25	96	49.67	6.66	98	21.50	3.55	98	8.73	1.04	82
GA, 4	77.41	10.72	91	47.10	6.52	96	22.05	3.22	89	8.26	0.98	77

TABLE III.—CONCENTRATION OF ALKALOIDS[a] IN STRAMONIUM PLANT PARTS

Treatment and Harvest Time, Wks.	Leaves-Tops		Stems		Roots	
	mg./Gm.	Control, %	mg./Gm.	Control, %	mg./Gm.	Control, %
Control, 0	4.1	...	4.3	...	2.9	...
SA, 0	3.8	93	3.2	74	2.8	97
GA, 0	4.4	107	4.6	107	1.8	62
Control, 2	3.9	...	2.8	...	0.95	...
SA, 2	4.3	110	2.8	100	1.4	147
GA, 2	3.2	82	3.0	107	0.95	100
Control, 4	5.3	...	2.2	...	1.9	...
SA, 4	5.2	98	2.4	109	2.8	147
GA, 4	5.0	94	1.9	86	1.7	89

[a] Total alkaloids were calculated as scopolamine.

TABLE IV.—TOTAL ALKALOID CONTENT[a] (MG.) OF STRAMONIUM

Treatment and Harvest Time, Wks.	Per Plant		Leaves-Tops		Stems		Roots	
	Total Alkaloids	Controls, %	Total Alkaloids	Controls, %	Total Alkaloids	Controls, %	Total Alkaloids	Controls, %
Control, 0	1.63	...	0.94	...	0.43	...	0.26	...
SA, 0	1.25	77	0.72	77	0.22	51	0.31	119
GA, 0	1.11	68	0.70	75	0.28	65	0.13	50
Control, 2	11.80	...	9.32	...	2.18	...	0.30	...
SA, 2	9.93	84	8.00	86	1.57	72	0.36	120
GA, 2	7.60	64	5.66	61	1.68	77	0.26	87
Control, 4	46.41	...	36.04	...	7.96	...	2.41	...
SA, 4	46.06	99	34.63	96	8.52	107	2.91	121
GA, 4	40.39	87	32.60	90	6.12	77	1.67	70

[a] Calculated from dry weight and alkaloid analyses data; per plant = leaves-tops + stems + roots.

at the terminal harvest. The roots of the SA group contained considerably more alkaloids than controls throughout the 4-week growth period, while the GA group had appreciably less total alkaloids (Table IV).

DISCUSSION AND CONCLUSIONS

The GA-treated seeds showed about a threefold increase over untreated seeds in germination percentage and also demonstrated a more rapid rate of germination. Treatment of belladonna seeds (10) and of hyoscyamus seeds (9) with GA has also promoted an earlier and more uniform emergence of seedlings and caused a higher percentage of seeds to germinate. Throughout the observation period the habit of these plants closely resembled the controls, except that they were slightly shorter than plants grown from untreated seed. Characteristic gibberellin effects, such as taller and spindlier plants, greater internodal elongation and a chlorotic appearance, have been reported (11,15–19) when plants were treated with GA. These effects were not observed in plants grown from seed treated with GA. It was noted from dry weight data that during the early stages of growth the seed treatment induced a deleterious effect on growth. Although these plants weighed less than controls at the final harvest the reduction in plant growth was not considered significant. This is in contrast to a generally beneficial effect usually reported for *Daturas* treated with GA (11, 15, 16, 18).

Inconsistent trends were observed regarding the concentration of alkaloids in the plant organs. Both increases and decreases, compared with the control group, were noted at the various harvests. The increased concentration in the aerial parts of the plant noted in some cases from treatment of seeds with GA differs from the reductions usually noted when *Daturas* are treated with the growth hormone (15, 16, 18). Although the total alkaloid content of the GA group was somewhat less than controls at the final harvest, the seed treatment with GA was considered beneficial since a greater and more uniform emergence of seedlings was obtained. The results obtained by the gibberellin-seed treatment in this experiment were generally similar to that in the study with belladonna seeds (10) which was being conducted concurrently with this one. However, a general increase in alkaloid formation was noted in belladonna grown from gibberellin-treated seeds.

Treatment of seeds with sulfuric acid induced about a twofold increase in germination percentage over untreated seeds. A more rapid and uniform emergence of seedlings was also noted in this group. The habit of this group approximated the controls. The growth rate of the SA group was considerably slower than the controls initially, however, at the final harvest the total dry weight of this group was about the same as controls. Both decreases and increases were noted in the concentration of alkaloids in the plant organs throughout the 4-week growth period. No significant difference in total plant alkaloids was noted in the SA group compared with controls at the final harvest. This differed from belladonna grown from seed treated with sulfuric acid since belladonna was stunted in growth and contained about 40% of the alkaloids of controls (10).

The application of various cold treatments to stramonium seeds did not cause an increase in germination. Actually the germination rate and total germination were considerably less in seeds treated by the constant freeze method and the freeze-thaw method. It was concluded that treating stramonium with gibberellic acid or sulfuric acid was beneficial since both the rate of germination and the number of seedlings germinating were significantly increased. The growth and total alkaloid content of the treated groups were not materially different from controls at the final harvest. These treatments are suggested when it is desirable to increase the germination of stramonium, especially when seeds are planted in late winter or early spring.

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Some Crystalline Modifications of the *tert*-Butylacetates of Prednisolone and Hydrocortisone

By JOHN A. BILES

Four phases of each of the two steroid esters have been identified. The phases have been characterized by using optical crystallography, X-ray powder diffraction, and infrared spectrometry. The optical crystallographic properties, melting points, densities, molar refractions, infrared spectra, and *d* distances are presented.

THE EFFECT of polymorphism on the activity of a drug has been emphasized in the recent literature. McCrone has stated (1) that the thermal stability of a drug depends on the polymorphic form and that "often the performance of a given compound can be improved through change to a different polymorphic form." Frederick said that occasionally a polymorph would be found whose properties were such that it would stand out in clinical effectiveness. He cautioned, however, that such a favorable polymorph might not be isolated for every compound studied (2). Higuchi, in discussing the physical chemical analysis of the percutaneous absorption process, stated that the highest thermodynamic potential possible for the penetrating agent must be used to obtain the maximum rate of penetration. Different crystalline modifications may exist having different free energies—thus different thermodynamic activities (3).

Two polymorphs of 6-α-methylprednisolone have been isolated (4). Ballard and Nelson prepared pellets of each of the two polymorphs and found that Form II of the steroid was absorbed after implants in rats 1.7 times as fast as Form I. They also reported that Form II was 1.2 times more soluble than Form I (5). There is evidence of four polymorphs of hydrocortisone alcohol, two of hydrocortisone acetate, and six crystalline modifications of cortisone acetate (6–8). Smakula and co-workers have reported one amorphous form and four polymorphs of 17-β-estradiol (9). Progesterone and ethynylestradiol are reported to be polymorphic (10, 11). Dickson, Page, and Rogers summarized the polymorphism of some additional steroids (12).

Cooper has stated that prednisolone *tert*-butylacetate (TBA) was at least trimorphic, and that this polymorphism presented a problem in formulation procedures (13). Rogers and Conbere reported that hydrocortisone TBA was dimorphic, while Williams reported that this ester was trimorphic (6, 14). The purpose of this communication is to report the crystalline modifications of the TBA of prednisolone and hydrocortisone. Each phase has been characterized by using three different tools. It was found that each tool could be successful in identifying the phase, but that the combination provided a powerful technique for identification and isolation of each of the crystalline modifications.

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EXPERIMENTAL

Crystallization of the Steroid Esters.—The two steroid esters were crystallized from various organic solvents at various temperatures. The crystals were collected following crystallization and dried. The crystals obtained were heated on the Kofler melting point block to determine phase changes and the melting point.

Hydrocortisone TBA was crystallized from various water-alcohol mixtures. Phase I, *i.e.*, the first modification obtained, was isolated from 20% ethanol. When the 90% alcoholic solution saturated with the steroid was allowed to evaporate at room temperature, Phase II was isolated. These methods of isolating the two phases have been described previously (14). A glass was isolated when hydrocortisone TBA was crystallized from chloroform. A white crystalline powder was obtained on scratching the glass. This was found to be a new crystalline modification and was designated as Phase III. A phase transition occurred when phase III was heated to 170°. In this manner Phase IV was isolated. It was also determined that when Phase I was heated on the Kofler block, a phase change occurred at approximately 140°. The phase obtained was identical with Phase IV.

The prednisolone TBA was crystallized from water-alcohol mixtures also. Phase I was isolated from 20% ethanol. Phase II was isolated from 90% ethanol. When the prednisolone ester was crystallized from 50% ethanol, three separate phases were distinguished by optical crystallographic procedures. Two of the modifications corresponded to Phases I and II. The third modification was termed Phase III; however, this phase could not be isolated in a

pure form. A fourth modification of the steroid was isolated when crystallized from acetone and termed Phase IV.

Determination of Solvates.—The amount of solvent bound to the steroid ester was determined by molar absorptivities and weight loss studies.

Determination of Physical Properties.—The procedures for the determination of the optical crystallographic properties, densities, and molar refractions have been described elsewhere (15, 16). The optical crystallographic properties of the modifications are recorded in Table I. The melting points, densities, and molar refractions of the phases are recorded in Table II. Photomicrographs of the phases are

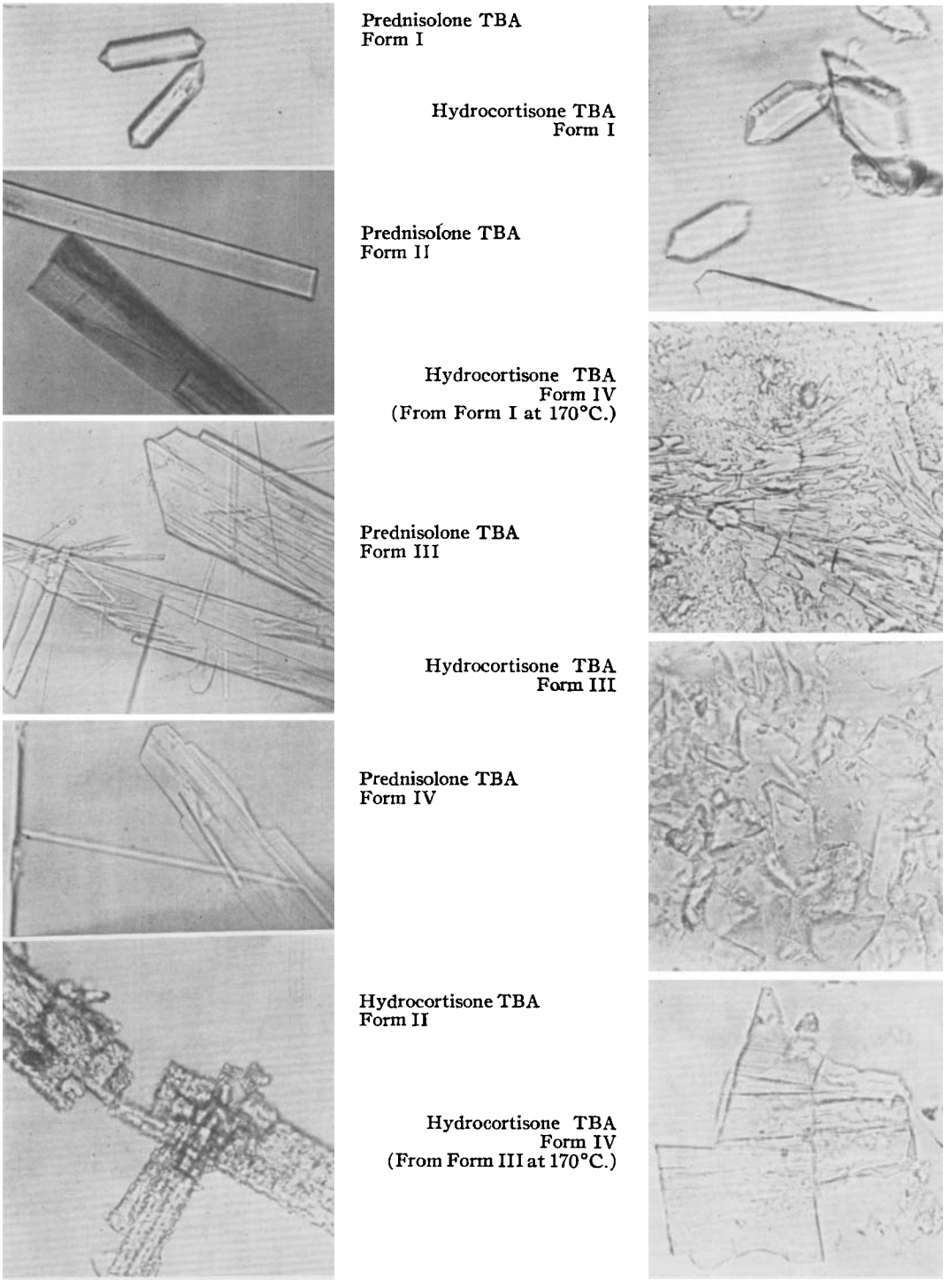
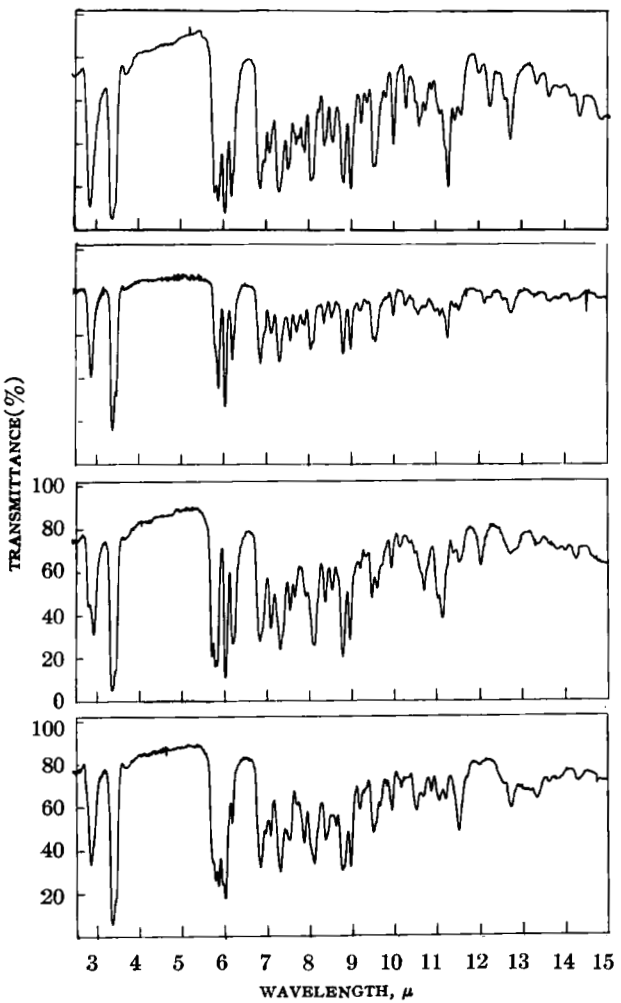


Fig. 1.—Photomicrographs showing the crystal modifications of TBA of prednisolone and hydrocortisone.



The infrared absorption spectra of prednisolone TBA crystallized from 20% ethanol. (Nujol mull). Phase I. ←

The infrared absorption spectra of hydrocortisone TBA crystallized from 95% ethanol. (Nujol mull). Phase II. →

The infrared absorptlon spectra of prednisolne TBA crystallized from 90% ethanol. (Nujol mull). Phase II. ←

The infrared absorption spectra of hydrocortisone TBA crystallized from chloroform. (Nujol mull). Phase III. →

The infrared absorption spectra of prednisolone crystallized from acetone. (Nujol mull). Phase IV. ←

The infrared absorption spectra of hydrocortisone TBA crystallized from chloroform and dried at 175°C. (Nujol mull). Phase IV. →

The infrared absorption spectra of hydrocortisone TBA crystallized from 20% ethanol. (Nujol mull). Phase I. ←

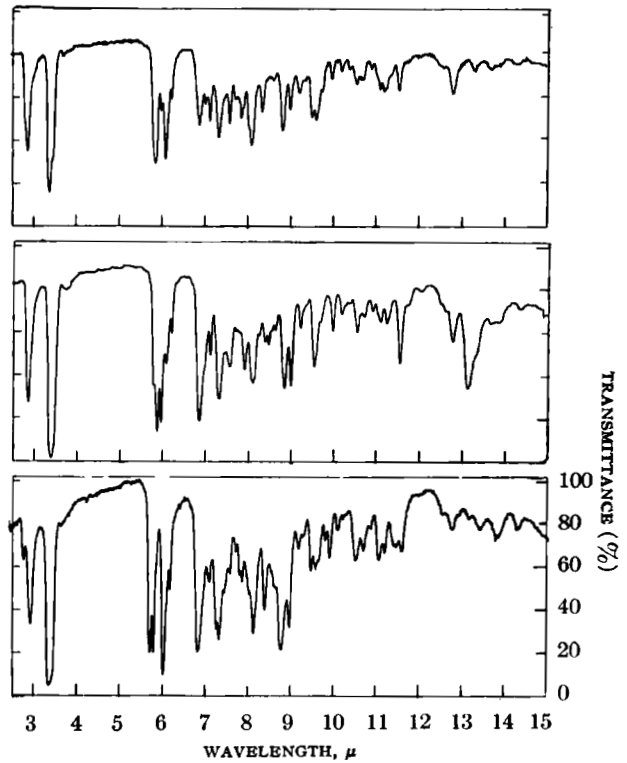


Fig. 2.—The infrared spectra of the crystal modifications of the TBA of prednisolone and hydrocortisone.

shown in Fig. 1. The photomicrographic techniques have been described previously (17).

Nujol mulls were prepared and the infrared absorption spectra were obtained using the Perkin-Elmer infracord spectrophotometer. The spectra of the crystalline modifications are illustrated in Fig. 2.

The X-ray powder analysis was made using a General Electric XRD-5 spectrometer.[1] The diffracted copper radiation (filtered by nickel) was detected by a quantum counter and was automatically recorded. The intensity of radiation was read as 2θ. The 2θ values were converted to d distances using the Bragg equation. The d distances are recorded in Table III.

RESULTS AND DISCUSSION

Four separate phases of the TBA of prednisolone and hydrocortisone have been isolated. One phase of prednisolone TBA was anhydrous; a second phase was isolated from 95% ethanol and found to be a monoethanol solvate. A third phase was identified but not isolated when crystallized from 50% ethanol. A fourth phase, crystallized from acetone, was found to be the hemisolvate form of the ester. When Phases II and IV of prednisolone TBA were dried in the Abderhalden at 140°, Phase I was obtained. The phase transition can also be observed on the Kofler block. Identification of the phases were determined by infrared and X-ray diffraction powder analyses and optical crystallographic analysis.

Phases I and II of hydrocortisone TBA were isolated by crystallizing from ethanol. Phase I was isolated when crystallized rapidly from ethanol, while Phase II was isolated on slow evaporation of the solvent. Both phases were found to be monoethanol solvates. Phase III of hydrocortisone TBA was isolated from a chloroformic solution. The phase was determined to be a hemichloroform solvate. When Phases I, II, and III were heated in the Abderhalden apparatus and on the Kofler block Phase IV was isolated. The latter phase is anhydrous.

The physical properties of all phases were studied. The melting points (uncorrected), densities, and molar refractions are reported in Table II. The optical crystallographic properties of the various phases are recorded in Table I. An outstanding feature of optical crystallography is the identification and characterization of crystalline substances in heterogeneous systems. Only with this method was Phase III of the prednisolone ester studied extensively.

The photomicrographs of the phases are shown in Fig. 1. The photomicrograph of Phase II of hydrocortisone TBA shows that the crystal solvate dissolved rapidly in the immersion oil. The same oil was used to immerse Phase I of the same ester. Thus, the rates of dissolution of the dimorphic phases in a mineral oil–bromonaphthalene immersion oil are different. Similar cleavage planes were observed of Phase IV of hydrocortisone TBA when Phases I or III were heated to 170°. The melting point of Phase I and resolidification were observed

[1] The infrared and X-ray spectra were determined in the Pharmacy Research and Development Laboratories, The Upjohn Co., Kalamazoo, Mich.

TABLE I.- OPTICAL CRYSTALLOGRAPHIC PROPERTIES OF THE CRYSTAL MODIFICATIONS

Crystal Modification	System	Crystal Habit	Optic Sign	Axial Angle	Optic Orientation[a]	Dispersion	Refractive Indexes $\alpha(\omega)$	$\beta(\epsilon)$	γ
Prednisolone TBA									
Phase I (Anhydrous phase)	Hexagonal	Columnar	+	0°	$\omega \parallel$ a $\epsilon \parallel$ c	None	1.552	1.572	...
Phase II (Monoethanol solvate)	Monoclinic	Columnar	+	56°	YY $\parallel$ b XX $\wedge$ c 27°	r>v	1.516	1.542	1.645
Phase III	Orthorhombic	Tabular	+	65°	XX $\parallel$ a YY $\parallel$ b ZZ $\parallel$ c	None	1.502	1.532	1.616
Phase IV (Hemiacetone solvate)	Orthorhombic	Tabular	+	31°	XX $\parallel$ a YY $\parallel$ c ZZ $\parallel$ b	v>r Strong	1.535	1.542	1.644
Hydrocortisone TBA									
Phase I (Monoethanol solvate)	Hexagonal	Columnar	+	0°	$\omega \parallel$ a $\epsilon \parallel$ c	None	1.541	1.565	...
Phase II (Monoethanol solvate)	Orthorhombic	Lamellar or prismatic	+	...	XX $\parallel$ a YY $\parallel$ c ZZ $\parallel$ b	r>v	1.505	1.526	...[b]
Phase III (Hemichloroform solvate)	....[c]	Tabular fragments	−	0°	...[c]	None	1.574	1.545	...
Phase IV (Anhydrous)	....[c]	Tabular fragments	+	37°	...[c]	r>v	1.525	1.534	1.623

[a] Crystallographic axes determined from crystal habit; a<b<c. [b] Could not determine because of phase change and solubility in oils. [c] Could not grow suitable crystals to determine system or optic orientation.

TABLE II.—MELTING POINT, DENSITY, AND MOLAR REFRACTIONS FOR CRYSTAL MODIFICATIONS

Crystal Modification	M. p., °C.[a]	Density	$\sqrt[3]{\alpha\beta\gamma}$ or $\sqrt[3]{\omega^2\epsilon}$	Exptl. M_{RD}	Calcd. M_{RD}
		Prednisolone TBA			
Phase I	244–249	1.210	1.559	122.40	121.76
Phase II	145[b]	1.239	1.566	132.87	134.54
Phase III	240–248	...[c]	1.549	...[c]	...[c]
Phase IV	226–230	1.254	1.573	130.55	129.83
		Hydrocortisone TBA			
Phase I	170[d]	1.194	1.549	135.18	135.04
Phase II	140[d]	...[e]	...[e]	...[e]	135.04
Phase III	145[d]	1.239	1.564	136.58	132.97
Phase IV	216–219	1.213	1.560	122.77	122.26

[a] Uncorrected. [b] Crystals darkened; possible phase change. Darkened crystals melted at 248–260°C. [c] Could not isolate to determine values. [d] Phase change. [e] Metastable phase. Could not determine all values.

under the microscope when using the Kofler block. No melting was observed when Phase III was converted to Phase IV by heating to 170°.

The X-ray powder patterns for the seven phases of the two steroid esters isolated were obtained using an XRD-5 spectrometer. The *d* distances are recorded in Table III. The first reflection for all phases, with one exception, corresponded to a distance of 14 + Å. Often this distance is indicative of the longest length of the unit cell. The first reflection which appeared for Phase III of the hydrocortisone ester corresponded to a distance of 22.64 Å.

TABLE III.—*d* DISTANCES FOR CRYSTAL MODIFICATIONS USING CU K-α RADIATION[a]

Prednisolone TBA Phase I	Prednisolone TBA Phase II	Prednisolone TBA Phase IV
14.97	14.97(s)[b]	14.80(s)
10.69	12.10	11.78(s)
8.65(s)	9.44(s)	10.16
7.50(s)	7.50	9.40
6.75	6.85	8.58
5.65(s)	6.01	7.56
5.32(s)	5.64	7.30
4.77	5.37	6.19
4.37	4.98	6.07
4.18	4.74(s)	5.90
4.02	4.63	5.78(s)
3.76	4.38	5.65
3.64	4.17	5.57
3.37	3.95	5.33
3.21	3.75	5.06(s)
3.02	3.58	4.39
...	3.22	4.29
...	...	4.23
...	...	3.90
...	...	3.87

Hydrocortisone TBA Phase I	Hydrocortisone TBA Phase II	Hydrocortisone TBA Phase III	Hydrocortisone TBA Phase IV
14.97	14.48	22.64(s)	14.24(s)
10.69	12.32	11.47(s)	12.62
9.64	9.50(s)	10.04(m)	11.23
8.61(s)	7.30	7.58	9.64
7.50(s)	6.75	7.19	7.72
6.36	5.95	5.89	6.60
5.65(s)	5.72	5.58	6.14
5.30(s)	5.28	5.50(m)	5.71(s)
4.76	4.77(s)	5.01(m)	5.48(s)
4.52	4.57(s)	4.67	5.24(s)
4.37	3.99	4.65	4.69
4.18	3.88	4.46	4.47
4.03	3.63	4.37	4.38
3.76	3.55	4.29	4.22
3.64	...	4.23	3.97
3.49	...	4.02	3.77
3.37	...	...	3.41
3.21	...	...	3.24
3.01	...	...	...

[a] Wavelength of 1.54051 Å. [b] (s) is strong and (m) is medium.

The infrared spectra for the phases are presented in Fig. 2. Since Phase III of the prednisolone ester could not be isolated in pure form, no spectra are submitted. The infrared spectra of the various phases are different and are of value for phase identification. The author observed that a given phase could be identified more easily using the XRD-5 spectrometer than using the infrared spectrometer. Gaebler, Parsons, and Beher have stated that X-ray diffraction methods are more effective than infrared methods in distinguishing compounds from mixtures (18). The hydroxyl stretching frequencies for the crystal phases varied between 3413 cm.$^{-1}$ for Phase IV of the prednisolone ester and 3534 cm.$^{-1}$ for Phase II of the hydrocortisone ester. Two peaks were observed for Phase IV of the hydrocortisone ester. One vibration was recorded at 3636 cm.$^{-1}$ which was indicative of a free hydroxyl group. The other vibration was recorded at 3448 cm.$^{-1}$, indicative of rather strong hydrogen bonding.

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Interaction of Urea and Thiourea with Benzoic and Salicylic Acids

By SANFORD BOLTON

Urea forms soluble complexes of low stability with benzoic acid in water, methanol, and dioxane. With salicylic acid, urea forms insoluble interaction products which can be isolated from the above solvents. On the basis of these results a procedure for the separation of salicylic acid from benzoic-salicylic acid mixtures is described. Thiourea forms only soluble complexes of low stability. The difference in the extent of interaction in the different solvent systems is explained on the basis of competing solute-solvent interactions, which can be translated into terms of solute solubilities.

ALTHOUGH in recent years much work has been reported on the formation of molecular complexes in pharmaceutical systems, the complexing agents used have often been toxic or medicinally potent substances. The desirability of investigating the complexing properties of nontoxic or less potent compounds is apparent. In the present study, urea, a comparatively innocuous drug, has been examined as a possible complexing agent.

Clathrates, or inclusion compounds of urea, have found useful application in the separation of closely related hydrocarbons (1–4) and mixtures of structurally related fatty acids (5, 6). Inasmuch as these urea inclusion complexes usually involve very small molecules such as CO_2 and H_2O_2 or straight chain hydrocarbons and their derivatives, it became of interest to study the interaction of urea solutions with more complex molecules where clathrate formation is not possible. Evidence of nonclathrate complexes of urea and pharmaceutically important substances has appeared in the literature. For example, aqueous solubilities of terramycin (7) and benzocaine (8) were shown to be increased in the presence of urea. Solid tetracycline-urea complexes have been prepared which have improved stability and solubility properties over the uncomplexed drug (9–11). Complexes of sulfonamides (12), wool fat alcohols (13, 14), quinoxaline (15), detergents (16–18), and barbituric acid derivatives (19) with urea have also been described.

In this report the interactions of urea and thiourea (chosen for purposes of comparison) with benzoic and salicylic acids have been followed by means of solubility studies. The high affinity of urea for water resulted in a low complexing tendency in this solvent. (See References 7 and 8.) Therefore, two nonaqueous solvents, methanol and dioxane, were also used. Although, in general, the degree of interaction was small in all cases, definite complexes were found. The difference of the solubility characteristics of the urea–benzoic acid and urea–salicylic acid complexes in the nonaqueous solvents suggested a possible separation procedure of salicylic acid from benzoic-salicylic acid mixtures. One approach to such a separation is described.

EXPERIMENTAL

Solubility Studies

A. Aqueous Solutions.—The interactions in aqueous solution were followed by determining acid solubility as a function of urea (thiourea) concentration. Excess benzoic (0.25 Gm.) and salicylic (0.625 Gm.) acids were equilibrated with 10 ml. of solutions of varying urea and thiourea concentrations by rotating the mixtures in capped vials for 18 hours in a constant temperature water bath at 30 ± 0.1°. All solutions were made 0.1*N* with H_2SO_4 to prevent dissociation of the acid species. The amount of acid in solution was determined concurrently by two methods: (*a*) titration of a clear aliquot with standardized NaOH solution; and (*b*) spectrophotometric examinations utilizing the peak for benzoic acid at 274 mμ and salicylic acid at 302.5 mμ.

B. Nonaqueous Solutions.—The interactions in methanol and dioxane were followed by determining acid solubility as a function of urea (thiourea) concentration and urea (thiourea) solubility as a function of acid concentration. Approximately 3 ml. of solution was equilibrated as in *A* for at least 24 hours in vials equipped with polyethylene-lined caps. The amounts of reagent in excess during the solubility studies are tabulated in Table I.

The mixtures were analyzed as follows. One milliliter of a clear aliquot was pipeted into a tared 10-ml. beaker, and the solvent was removed by evaporation with the aid of gentle heat. The residue was weighed and acid content was determined by titration with a standardized NaOH solution. The urea content could then be determined by difference. (Since the solutions often contained large concentrations of reactants, it was very difficult to measure total solution volume accurately. This presented no problem when only soluble interaction species were formed, as the method of analysis gave

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TABLE I.—COMPONENTS IN NONAQUEOUS SYSTEMS

System	Wt. of Component in Excess, Gm.
Salicylic acid–urea–methanol	Salicylic acid–1.35 urea–0.70
Salicylic acid–urea–dioxane	Salicylic acid–1.40 urea–0.20
Benzoic acid–urea–methanol	Benzoic acid–2.0 urea–1.0
Benzoic acid–urea–dioxane	Benzoic acid–2.0 urea–0.15
Salicylic acid–thiourea–methanol	Salicylic acid–2.0 thiourea–0.40
Salicylic acid–thiourea–dioxane	Salicylic acid–2.0 thiourea–0.75
Benzoic acid–thiourea–methanol	Benzoic acid–2.0 thiourea–0.40
Benzoic acid–thiourea–dioxane	Benzoic acid–2.0 thiourea–0.75

molar concentrations directly; i.e., the concentration of complexing agent added equalled the concentration found in solution. The points on the graphs after the appearance of an insoluble species are approximations, since changes in solution volume during the precipitation process could not be determined.)

RESULTS

Interaction Studies

A. Aqueous Solution.—Figure 1 illustrates the effect of urea and thiourea on the solubilities of benzoic and salicylic acids in water. Only in the case of the salicylic acid–urea interaction did an insoluble interaction product appear before urea or thiourea saturation. Along the first plateau region of this curve an insoluble species appeared; along the immediately following descending portion the precipitate showed acid-urea ratios between 1–1 and 2–1. Calculation of the acid-urea ratio from the phase diagram according to Higuchi and Lach (20) suggested formation of a 1–1 complex which could not be reconciled with the analysis of the insoluble phase in this region of the curve. The form of the following portion of the curve suggested that a new insoluble species was being formed, and analysis of the precipitate in this region corresponded to a 1–1 complex. After this plateau region the apparent solubility of acid increased slowly, although the insoluble phase still consisted of a 1–1 species. The high concentrations of urea at the latter portions of the curve undoubtedly were at least partially responsible for the unusual solubility pattern of the acid and no effort was made to rationalize the curve in this region.

Because of the high concentrations of complexing agent used and the uncertainty of the nature of the interaction products, stability constants for the interactions were not calculated. It is apparent by inspection of Fig. 1, however, that the stability of salicylic acid complexes were stronger than the benzoic acid complexes, and the thiourea complexes were stronger than the urea complexes.

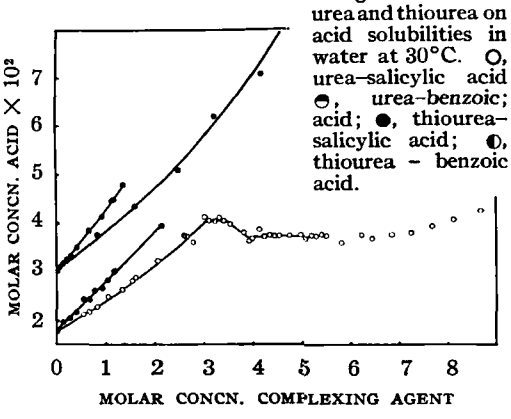

Fig. 1.—Effect of urea and thiourea on acid solubilities in water at 30°C. ○, urea–salicylic acid ◒, urea–benzoic; acid; ●, thiourea–salicylic acid; ◐, thiourea – benzoic acid.

B. Nonaqueous Solutions.—Figures 2, 3, and 4 illustrate the results obtained in methanol and dioxane. Figure 2 shows the effect of urea on the acid solubilities in these solvents. Thiourea showed no complexing tendencies under these conditions; these data are not included in the plot. The solubilities of the acids decreased slightly in the presence of thiourea. A slight decrease or lack of change of acid solubility did not necessarily indicate a lack of interaction in these systems. At the high solute concentrations used in these studies, if no interaction takes place between the solutes, we might expect a competition for solvent molecules and a resultant substantial decrease in solubility. In fact, this mutual "squeezing out" of the solute molecules probably occurred to some extent in most of the systems in this study. Urea formed a 1–1 insoluble complex with salicylic acid in both methanol and dioxane. The final plateau regions in these curves indicate saturation with respect to urea as well as complex and the systems are invariant. The plateaus formed in the benzoic acid–urea systems were due to urea saturation; only soluble species were formed in these systems. It is apparent that urea complexed more strongly with benzoic acid in dioxane than methanol.

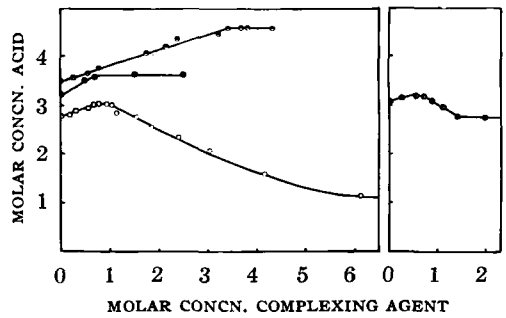

Fig. 2.—Effect of urea on acid solubilities in methanol and dioxane at 30°C. ○, urea–salicylic acid–methanol; ◒, urea–benzoic acid–methanol; ◐, urea–salicylic acid–dioxane; ●, urea–benzoic acid–dioxane.

The effect of the acids on urea and thiourea solubility in dioxane and methanol is shown in Figs. 3 and 4. These studies further establish the nature of the interactions, especially those in dioxane where urea and thiourea solubilities were comparatively small. The low interaction tendency of thiourea in methanol was confirmed. However, in dioxane, the solubility of thiourea was increased

to a small extent in the presence of the acids; salicylic acid proved to be the slightly stronger complexing agent (Fig. 4). Also the comparatively strong interaction of urea with the acids in dioxane is clearly illustrated in Fig. 4. Again, the final invariant regions in these curves are caused by saturation of the system by complexing agent.

Comparison of the extent of interaction, somewhat obscure in most cases in the acid-saturated system (Fig. 2), can be made from examination of Figs. 3 and 4. The urea–salicylic acid complex was stronger in dioxane than methanol and the urea–salicylic acid complex was stronger than the urea–benzoic acid complex in dioxane.

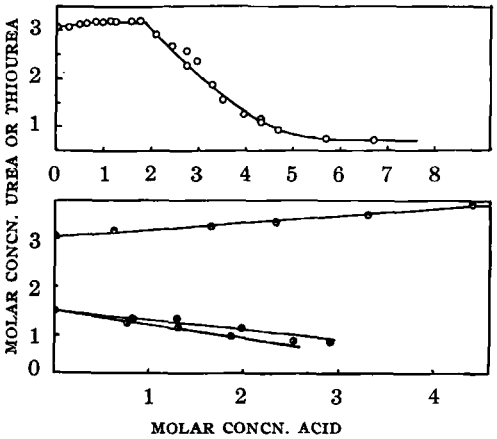

Fig. 3.—Effect of acid on urea and thiourea solubilities in methanol at 30°C. ○, urea–salicylic acid; ◓, urea–benzoic acid; ◑, thiourea–salicylic acid; ●, thiourea–benzoic acid.

Separation of Salicylic Acid from Salicylic-Benzoic Acid Mixtures

A procedure for a separation of salicylic acid from mixtures with benzoic acid was designed based on the low solubility of the urea–salicylic acid complex compared to the urea–benzoic acid complex in methanol. Methanol, rather than dioxane, was chosen as the solvent for the procedure because the low solubility of urea in dioxane reduced the amount of salicylic acid which it can precipitate. (The system, urea–salicylic acid–dioxane, became invariant due to urea saturation with almost 3*M* salicylic acid in solution, while the invariance point in methanol left only 1*M* salicylic acid in solution. See Fig. 2. Also, because of the greater solubility of urea in methanol, urea solubilized benzoic acid more in methanol than in dioxane.

The method of separation was as follows. One gram of a mixture of salicylic and benzoic acids was shaken with 1 ml. of methanol and $^2/_3$ Gm. of urea for approximately 1 hour at 30°. The resultant precipitate was filtered and sucked dry under

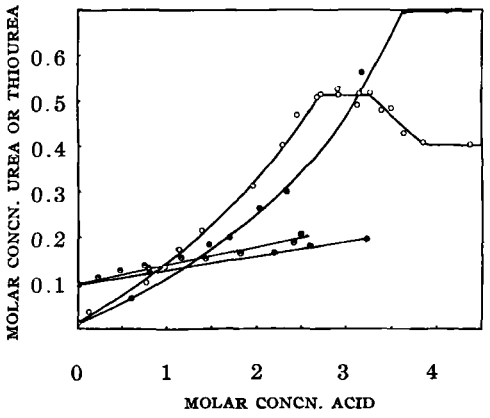

Fig. 4.—Effect of acid on urea and thiourea solubilities in dioxane at 30°C. ○, urea–salicylic acid; ●, urea–benzoic acid; ◓, thiourea–salicylic acid; ◐, thiourea–benzoic acid.

vacuum. The dried precipitate was weighed and mixed with 15 ml. of water per 2 Gm. of precipitate. This slurry was stirred with a magnetic stirrer for 5–7 minutes to dissolve the urea, and the remaining solid was collected in a sintered-glass funnel and dried. The salicylic acid content of this residue was assayed by measuring the color produced with ferric chloride using a Bausch and Lomb spectronic 20 colorimeter (21). Further salicylic acid purity can be achieved by repeating the process. (See Table II.)

Table II shows the results of this separation for two acid mixtures. No effort was made to recover salicylic acid quantitatively, and the column headed *Ppt. Recovered* is merely for information. More quantitative yields could be obtained by repeating the process on the alcohol soluble portion.

DISCUSSION

As mentioned before, no effort was made to calculate equilibrium constants since the exact nature of the interactions was not known. However, in order to have a basis on which to compare the extent of interaction in the different systems, values were calculated according to Higuchi and Zuck (22) from the initial portions of the curves as if 1–1 complexation were occurring. Table III shows the results of these calculations.

The effect of solvent on the extent of interaction may be explained on the basis of competitive reactions between solute and solvent molecules, *i. e.*, the greater the interaction of the solutes with the solvent, the lower will be the complexing tendency between solute molecules. This effect can be translated in terms of solubility which is a measure of solute-solvent interaction forces. Since the solubilities of the two solutes should be a factor in deter-

TABLE II.—RECOVERY OF SALICYLIC ACID FROM BENZOIC ACID–SALICYLIC ACID MIXTURE

Acid Mixture, Wt. and Compn.	Ppt. Recovered, Wt. and Compn.	Wt. and Compn. of Ppt. Recovered (process repeated on Ppt. in Col. 2).
15 Gm.–33% salicylic acid–67% benzoic acid	2.4 Gm.–78% salicylic acid	1.5 Gm.–98.5% salicylic acid
20 Gm.–50% salicylic acid–50% benzoic acid	6 Gm.–98% salicylic acid	3.7 Gm. 99.5% salicylic acid

TABLE III.—APPARENT 1–1 STABILITY CONSTANTS

Solvent	Interacting Species	Approximate K_1 (Complex)/(Urea) (Acid)
Water	Urea–benzoic acid	0.2
	Urea–salicylic acid	0.3
	Thiourea–benzoic acid	0.33
	Thiourea–salicylic acid	0.55
Methanol	Urea–benzoic acid	0.12
	Urea–salicylic acid	0.15
	Thiourea–benzoic acid	—
	Thiourea–salicylic acid	—
Dioxane	Urea–benzoic acid	5
	Urea–salicylic acid	7
	Thiourea–benzoic acid	0.32
	Thiourea–salicylic acid	0.47

mining the magnitude of the interaction, it is well to know the solubilities of all the species involved. In the present studies, solubilities can be conveniently obtained from the figures, except for urea and thiourea in water, which are approximately 10 *M* and 2 *M*, respectively (23). It is apparent from these considerations that the degree of interaction seems to be directly related to the solubilities of the interacting species. The acid-urea interactions are strongest in dioxane > water > methanol. Considering that all species are extremely soluble in methanol, the small reactivity in this solvent would be expected. A further look at the dioxane and water systems reveals that in each solvent one component is very soluble, while one component is relatively insoluble; the acids are insoluble in water, while urea is insoluble in dioxane. A comparison of actual solubility values shows that the solubilities of both the insoluble and soluble components in water are greater than both values in dioxane. This, it seems, would account for the greater complexing tendency in dioxane. Exactly analogous considerations apply and hold for the thiourea interactions. It is interesting that the degree of interaction for thiourea is very similar in both water and dioxane and the products of thiourea and acid solubilities are also very close in both solvents.

Similar observations have been made by Marvel and Lemberger (24) and Gans and Higuchi (25). In both cases, differences in complexing tendencies in different solvents were attributed to competing solvent-solute interactions. Unfortunately, the solubilities of the complexing agents in the solvents studied were not determined and an analysis similar to the above one cannot be made.

The toxic nature of thiourea and its low complexing tendencies in these nonclathrate type systems suggest a limit to its possible usefulness. On the other hand, results presented here and past reports of urea complexes strongly recommend further investigation of urea as a complexing agent in pharmaceutical systems. Its low complexing tendency in water should be more than compensated by its high solubility and comparative nontoxicity.

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Mechanisms of Steroid Oxidation by Microorganisms III

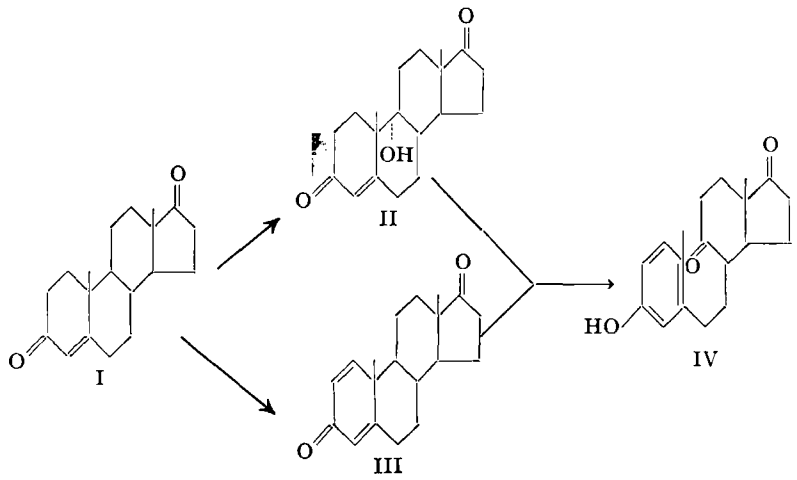
Enzymatic Mechanism of Ring A Aromatization

By CHARLES J. SIH and A. M. RAHIM

Evidence is presented to show that the aromatization of 9α-hydroxyandrost-4-en-3,17-dione and 19-hydroxyandrost-4-en-3,17-dione involves a 1,2-dehydrogenation by the steroid 1-dehydrogenase to afford vinylogs of β-hydroxyketones which may undergo spontaneous nonenzymic rearrangement (reverse aldolization) to give their respective phenols. This mode of aromatization differs from the human placental system in that it requires a typical flavoprotein electron acceptor for activity; oxygen and $NADPH_2$ are not required for aromatization. Also, the relative rates of oxidation follows the order 19-norandrost-4-en-3,17-dione > 19-oxoandrost-4-en-3,17-dione > 19-hydroxyandrost-4-en-3,17-dione.

ONE PATHWAY of steroid degradation by microorganisms involves a 9α-hydroxylation, followed by a 1,2-dehydrogenation (or vice versa) with the formation of a 9,10-seco phenol (1). The degradation of androst-4-en-3,17-dione could thus be visualized as follows: androst-4-en-3,17-dione (I) → 9α-hydroxyandrost-4-en-3,17-dione (II) or androsta-1,4-diene-3,17-dione (III) → 3-hydroxy-9,10-seco-androsta-1,3,5(10)-triene-9,17-dione (IV).

We have previously postulated that the microbial aromatization of 9α-hydroxyandrost-4-en-3,17-dione and 19-hydroxyandrost-4-en-3,17-dione probably involved a 1,2-dehydrogenation followed by reverse aldol condensation, their results were obtained solely from the intact microorganism. A preliminary communication (3) on the enzymatic mechanism of this aromatizing reaction has appeared. In view of the importance of this mechanism we wish to record in detail



Scheme I

This mechanism of Ring A aromatization is of biochemical interest since it bears close similarity to the formation of estrogens from androgens in mammals (2). Although Dodson and Muir (1) our examinations of reactions using partially purified enzyme preparations which are significant in establishing and confirming the proposed mechanism of Dodson and Muir.

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EXPERIMENTAL

Materials and Methods

Cytochrome *c*, nicotinamide-adenine dinucleotide (NAD), nicotinamide-adenine dinucleotide phosphate (NADP), reduced nicotinamide-adenine dinucleotide ($NADH_2$), and reduced nicotinamide-

adenine dinucleotide phosphate ($NADPH_2$) were obtained from Sigma Chemical Co. Phenazine methosulfate was purchased from the Aldrich Chemical Co. Whatman No. 1 paper from H. Reeve Angel and Co. was used for paper chromatography. 2,3 - Dichloro - 5,6-dicyanobenzoquinone (DDQ) was purchased from the Ealing Corp. 9α-Hydroxyandrost-4-en-3,17-dione was prepared according to the method of Sih (4). 9α-Fluorohydrocortisone was synthesized according to the method of Fried and Sabo (5). All melting points were uncorrected and were determined in open soft-glass capillaries. Values of $[\alpha]_D$ have been approximated to the nearest degree. Ultraviolet absorption spectra were determined on a Cary model 11 MS recording spectrophotometer. Infrared spectra were recorded on a Beckman IR 5A double beam infrared recording spectrophotometer. Microanalyses were carried out by Mr. J. Alicino of Metuchen, N. J. Protein was estimated by the method of Gornall, *et al.* (6). Phenolic steroids were detected on paper chromatograms by spraying with diazotized sulfanilic acid (7). Spectrophotometric measurements were made on a Beckman DU spectrophotometer equipped with a Gilford multiple sample absorbance recorder model 2000 (8).

The conditions and the procedure used for the growth of *N. restrictus*, the purification of the steroid 1-dehydrogenase, and the details of the phenazine methosulfate-cytochrome *c* assay have been described previously (9). The solvent system for paper chromatography consisted of toluene-propylene glycol (10) and the R_f values of the steroids used in this work are shown in Table I. Δ^4-3-Oxo and $\Delta^{1,4}$-3-oxo steroids were assayed quantitatively after their elution from the paper chromatogram; their absorbances at 240 mμ were taken as a measure of their concentration (9). Phenolic steroids were assayed spectrophotometrically by measuring the increase in absorbance at 280 mμ (11).

Enzymatic Transformation of 9α-Hydroxyandrost-4-en-3,17-dione (II) into 3-Hydroxy-9,10-seco - androsta - 1,3,5(10) - triene - 9,17 - dione (IV).—A 100-mg. quantity of phenazine methosulfate and 50,000 units of 1-dehydrogenase (specific activity 1000) in a total volume of 400 ml. of 0.03 *M* phosphate buffer, pH 7.0, was added to 200 mg. of II. After a 4-hour incubation, the reaction was terminated by the addition of 6 *N* HCl and the protein precipitate was removed by filtration. The filtrate was extracted with three 150-ml. portions of chloroform, dried over sodium sulfate, and taken down to dryness. The residue was taken up in 75 ml. of benzene-ether (1:1) and extracted with three 35-ml. portions of 5% NaOH. The aqueous layer was acidified and extracted with 40-ml. portions of chloroform three times; the chloroform layer was dried over sodium sulfate and concentrated to dryness to give 168 mg. of residue. Two recrystallizations from acetone-petroleum ether afforded 94 mg. of IV, m.p. 122–124°; $[\alpha]_D^{25}$ +98° in chloroform (c, 0.9); $\lambda_{max.}^{alc.}$ 280 mμ (ε 2700); $\lambda_{max.}^{Nujol}$ 2.95, 5.75, 5.86, 6.23, and 6.66μ, identical to an authentic specimen.

Anal.—Calcd. for $C_{19}H_{24}O_3$: C, 75.97; H, 8.05. Found: C, 76.23; H, 8.53.

Enzymatic Dehydrogenation of 9α-Fluorohydrocortisone.—To 100 mg. of 9α-fluorohydrocortisone was added 50 mg. of phenazine methosulfate and 50,000 units of 1-dehydrogenase (specific activity 700) in a total volume of 500 ml. of 0.03 *M* phosphate buffer, pH 7.0. After incubation for 16 hours, the reaction was terminated by the addition of HCl and the mixture was extracted with 100 ml. of methylisobutyl ketone three times. The combined methylisobutyl ketone extract was dried over sodium sulfate and concentrated to dryness to give 89 mg. of residue. Two recrystallizations from ethanol gave 65 mg. of crystals, m.p. 265–269° dec., identical in all respects (mixed melting point and infrared spectrum) with an authentic sample.

Reaction of 9α-Hydroxyandrost-4-en-3,17-dione with 2,3 - Dichloro - 5,6 - dicyanobenzoquinone.—A 500-mg. quantity of DDQ was added to 400 mg. of 9α - hydroxyandrost - 4 - en - 3,17 - dione in 20 ml. of dioxane. The mixture was refluxed for 16 hours under nitrogen. On cooling, the hydroquinone separated and filtered off. The filtrate was diluted with an equal volume of chloroform and poured onto an acid washed alumina column (20 Gm.). Elution with chloroform afforded 20 mg. of a compound, m.p. 120–122°, $\lambda_{max.}^{alc.}$ 280 mμ (ε 2600), whose infrared spectrum was identical to an authentic sample of IV.

Enzymatic Conversion of 19-Hydroxyandrost-4-en-3,17-dione into Estrone.—To 20 mg. of 19-hydroxyandrost-4-en-3,17-dione was added 10 mg. of phenazine methosulfate and 5000 units of 1-dehydrogenase (specific activity 1000) in a total volume of 100 ml. of 0.03 *M* phosphate buffer, pH 7.0. After a 12-hour incubation, the reaction was terminated by the addition of 6 *N* HCl and extracted with 50-ml. portions of chloroform three times. The combined chloroform extract was dried over sodium sulfate and taken down to dryness. Two recrystallizations from chloroform-methanol afforded 10 mg. of crystals, m.p. 260–262°, identical in all respects (melting point, mixed melting point, and infrared spectrum) with an authentic specimen of estrone.

In a separate experiment 1 mg. of 19-hydroxyandrost-4-en-3,17-dione was mixed with 500 mcg. of phenazine methosulfate and 1000 units of 1-dehydrogenase (specific activity 900) in a total volume of 4 ml. of 0.03 *M* phosphate buffer, pH 7.0. After a 2-hour incubation, the mixture was distilled and the distillate gave a positive reaction for the presence of formaldehyde (12). By omitting the 19-hydroxyandrost-4-en-3,17-dione from the reaction mixture, the distillate gave no purple color.

TABLE I.—*Rf* VALUES OF STEROIDS IN THE TOLUENE–PROPYLENE GLYCOL SYSTEM

Compd.	*Rf*
Androst-4-en-3,17-dione	0.90
9α-Hydroxyandrost-4-en-3,17-dione	0.35
Androsta-1,4-diene-3,17-dione	0.82
Androst-4-en-3-one,17β-ol	0.45
Androsta-1,4-diene-3-one-17β-ol	0.40
19-Hydroxyandrost-4-en-3,17-dione	0.10
19-Oxoandrost-4-en-3,17-dione	0.75
19-Norandrost-4-en-3-one-17β-ol	0.40
3-Hydroxy-9,10-seco-androsta-1,3,5(10)-triene-9,17-dione	0.33
3-Hydroxyestra-1,3,5-triene-17-one	0.37
9,10-Seco-androst-4-en-3,9,17-trione	0.85

RESULTS

In a previous paper we have reported the conversion of 9α-hydroxyandrost-4-en-3,17-dione into the 9,10-seco phenol (IV) by crude cell-free extracts of *N. restrictus* (13). Since phenazine methosulfate was required for this transformation, it was suspected that the steroid 1-dehydrogenase was involved in this conversion. Figure 1 shows that, using partially purified steroid 1-dehydrogenase (tenfold), a similar conversion could be obtained, evidenced by the rapid disappearance of absorbance around 240 mμ, accompanied by an increase in absorbance at 280 mμ. Thus all subsequent experiments were performed using this partially purified enzyme preparation. To substantiate further the participation of the steroid 1-dehydrogenase in this aromatization reaction, several quinones were tested for their ability to serve as electron acceptors because steroid 1-dehydrogenase has been implicated as a flavoprotein. Table II shows that the relative efficiency of these quinones as electron acceptors were the same with either androst-4-en-3,17-dione or 9α-hydroxyandrost-4-en-3,17-dione as the substrate. Figure 2 shows that the pH optimum of this aromatization reaction is around 9.0, identical to that of the oxidation of androst-4-en-3,17-dione. Because of the difficulty in preparing 9α-acetoxyandrost-4-en-3,17-dione and microorganisms contain esterases capable of hydrolyzing acetoxyl groups, 9α-fluorohydrocortisone was incubated and was readily converted into 9α-fluoroprednisolone by the same enzyme system. (See *Experimental.*)

Two possible mechanisms of this Ring A aromatization reaction involving the steroid 1-dehydrogenase were apparent and these can be represented as

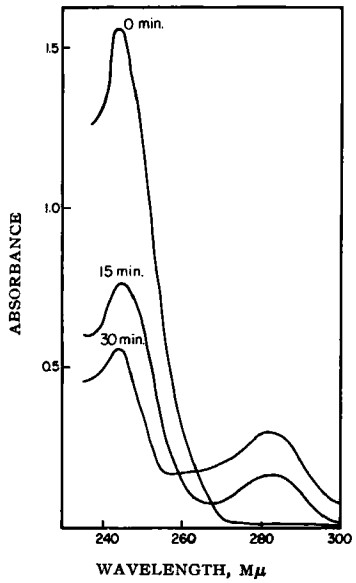
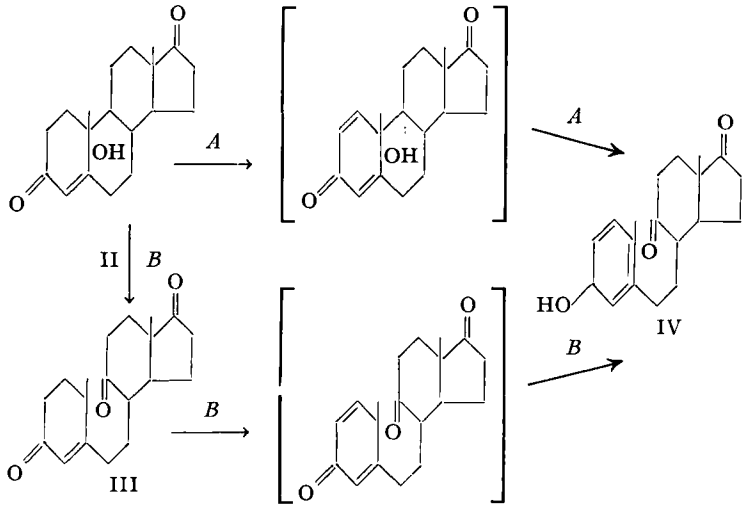


Fig. 1.—Oxidation of 9α-hydroxyandrost-4-en-3,17-dione to 3-hydroxy-9,10-seco-androsta-1,3,5(10)-triene-9,17-dione by purified steroid 1-dehydrogenase. The reaction mixture consisted of 600 mcg. of 9α-hydroxyandrost-4-en-3,17-dione, 500 mcg. of phenazine methosulfate and 25 units of 1-dehydrogenase (specific activity 1000) in a total volume of 4 ml. of 0.03 *M* phosphate buffer, pH 7.0. At the indicated time intervals the reaction was terminated by the addition of HCl and extracted with 1 ml. of chloroform. After separation of the layers by centrifugation, 0.2 ml. of the chloroform layer was diluted with 2.8 ml. of chloroform.



Scheme II

Mechanism *A* involves the introduction of a 1,2-double bond to form a vinylog of a β-hydroxyketone which could undergo facile rearrangement (reverse aldolization) to give a 9,10-seco phenol (IV). Mechanism *B* involves the abstraction of the 9α-hydroxyl proton to yield 9,10-seco-androst-4-en-3,9,17-trione (III); introduction of a 1,2-double bond into the latter compound affords a $\Delta^{1,4}$-dienone system which could also undergo chemical rearrangement to give a 9,10-seco phenol (IV). In order to distinguish these two mechanisms, 9α-hydroxyandrost-4-en-3,17-dione and 9,10-seco-androst-4-en-3,9,17-trione were incubated with the steroid 1-dehydrogenase. Figure 3 shows that the former compound was readily oxidized whereas 9,10-seco-androst-4-en-3,9,17-trione was not. This experi-

ment eliminates the latter compound as a possible intermediate and makes Mechanism *B* an unlikely one.

Table III shows that in the absence of phenazine methosulfate little if any 9α-hydroxyandrost-4-en-3,17-dione was metabolized. The slight decrease is probably due to the contamination of a trace amount of the natural electron acceptor. Addition of NAD and NADP did not stimulate the rate of metabolism of 9α-hydroxyandrost-4-en-3,17-dione, whereas on addition of phenazine methosulfate a rapid disappearance was observed.

On paper chromatograms we have been unable to detect the presence of 9α-hydroxyandrosta-1,4-diene-3,17-dione; the first demonstrable product has been the 9,10-seco phenol even when careful kinetic studies were made within the pH range of 4.8 to 9.0. Although it is well known that vinylogs of β-hydroxyketones undergo rearrangement very easily even by ordinary chemical means, methyl-3,11-dioxo-14,19-dihydroxy-$\Delta^{1,4}$, 14β-etiadienate has been obtained in very small yields as a by-product of a chemical reaction (14). This encouraged us to attempt to synthesize 9α-hydroxyandrosta-1,4-diene-3,17-dione by using mild chemical methods. However, when 9α-hydroxyandrost-4-en-3,17-dione was refluxed with DDQ, only 9,10-seco phenol (IV) was obtained in low yields; we were unable to detect the presence of the desired product on paper chromatograms.

Because this aromatization reaction bears close similarity to the mammalian conversion of androgens into estrogens, several of the intermediates in the mammalian pathway were incubated with the microbial system. 19-Hydroxyandrost-4-en-3,17-dione (V) was converted into estrone (VI). (See *Experimental.*) The distillate of the reaction mixture gave a positive test for the presence of formaldehyde (12), but the ratio of estrone to formaldehyde has been erratic and varied from experiment to experiment. In the absence of an electron acceptor such as phenazine methosulfate, very little 19-hydroxyandrost-4-en-3,17-dione (V) was metabolized. $NADH_2$ and $NADPH_2$ gave no stimulation (Table IV). 19-Oxoandrost-4-en-3,17-dione was also transformed into estrone by the same enzyme preparation; no attempt was made to determine the one-carbon fragment. Figure 4 shows the relative rates of oxidation follows the order: androst-4-en-3,17-dione>19-norandrost-3-en-3,17-dione>19-oxoandrost-4-en-3,17-dione>19-hydroxyandrost-4-en-3,17-dione. These rates were identical when the same experiment was carried out under anaerobic conditions.

TABLE II.—EFFECT OF DIFFERENT ELECTRON ACCEPTORS ON THE METABOLISM OF 9α-HYDROXYANDROST-4-EN-3,17-DIONE AND ANDROST-4-EN-3-ONE-17-OL[a]

Electron Acceptor	Relative Rate: 9α-Hydroxyandrost-4-en-3,17-dione	Relative Rate: Androst-4-en-3-one-17β-ol
1,2-Naphthoquinone	100	100
Phenazine methosulfate	83	86
Menadione	62	65
1,4-Naphthoquinone	47	51
2,6-Dichlorophenol indophenol	50	48
2,3-Dichloro-5,6-dicyanobenzoquinone	30	34

[a] The reaction system contained 1.5 μm. of 9α-hydroxyandrost-4-en-3,17-dione or androst-4-en-3-one-17β-ol, dissolved in 0.1 ml. of dimethylformamide, 1.5 μm. of various electron acceptors, and 20 units of enzyme (specific activity 500) in a total volume of 4.0 ml. of 0.03 *M* phosphate buffer, pH 7.0. After 10 minutes, the reaction products 3-hydroxy-9,10-seco-androsta-1,3,5(10)-triene-9,17-dione and androsta-1,4-diene-3-one-17β-ol were assayed as in Fig. 2.

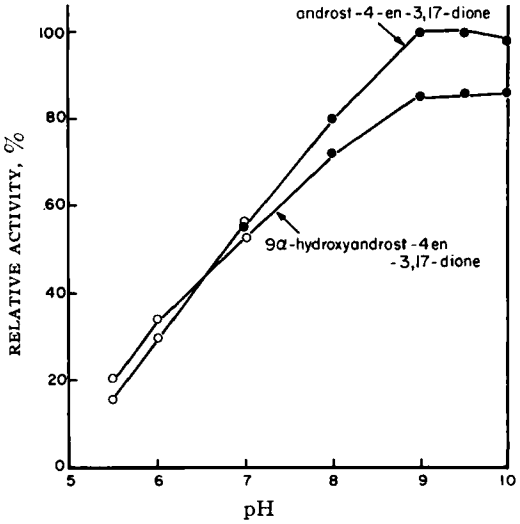
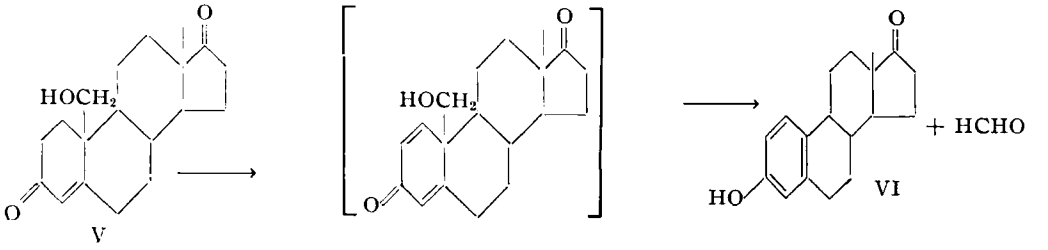


Fig. 2.—Effect of pH on the rate of oxidation of androst-4-en-3,17-dione and 9α-hydroxyandrost-4-en-3,17-dione. Key: O——O, 0.03 *M* phosphate; ●——●, 0.03 *M* tris (hydroxymethyl) aminomethane buffer. The reaction systems contained 1 mg. of androst-4-en-3,17-dione or 9α-hydroxyandrost-4-en-3, 17-dione, 500 mcg. of phenazine methosulfate, and 25 units of 1-dehydrogenase (specific activity 800) in a total of volume of 4 ml. of 0.03 *M* phosphate or Tris buffer. After 10 minutes, the reaction was terminated by acidification; androsta-1,4-diene-3,17-dione was assayed by the paper chromatographic method, and 9,10-seco phenol (IV) was assayed as in Fig. 1.



Scheme III

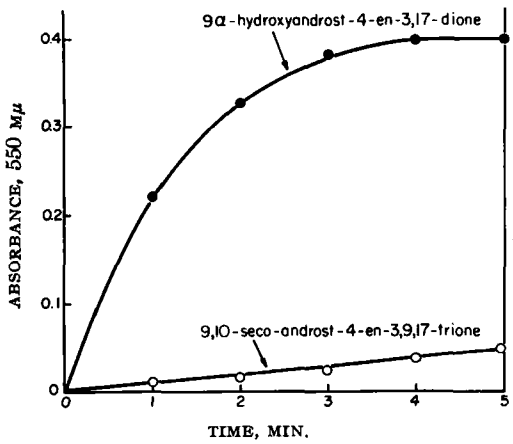


Fig. 3.—Reaction of 9α-hydroxyandrost-4-en-3,-17-dione and 9,10-seco-androst-4-en-3,9,17-trione with purified 1-dehydrogenase. The assay system contained 2 mg. of cytochrome *c*, 100 mcg. of steroid in 0.1 ml. of methanol, and 25 units of enzyme (specific activity 1000) in a total volume of 3 ml. of 0.03 *M* phosphate buffer, pH 7.0. The reaction was initiated by the addition of 0.1 ml. of a 1% phenazine methosulfate solution.

TABLE III.—EFFECT OF PHENAZINE METHOSULFATE ON METABOLISM OF 9α-HYDROXYANDROST-4-EN-3,17-DIONE[a]

Additions	9α-Hydroxyandrost-4-en-3,17-dione, μm. 20 min.	45 min.
None	1.59	1.36
NAD (1.5 μm.)	1.57	1.43
NADP (1.5 μm.)	1.56	1.48
Phenazine methosulfate (1.5 μm.)	0.03	0.00

[a] The reaction mixture contained 1.65 μm. of 9α-hydroxyandrost-4-en-3,17-dione in 0.2 ml. of dimethylformamide and 40 units of enzyme (specific activity 1000) in a total volume of 4 ml. of 0.03 *M* phosphate buffer, pH 7.0. The reaction was stopped at the indicated time intervals by the addition of 1 ml. of chloroform; 9α-hydroxyandrost-4-en-3,17-dione was determined by the paper chromatographic method.

DISCUSSION

The results described show that the conversion of 9α-hydroxyandrost-4-en-3,17-dione into 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione requires the presence of an electron acceptor characteristic of flavoproteins (15); the relative efficiency of these electron acceptors follows the same order regardless of whether androst-4-en-3,17-dione or 9α-hydroxyandrost-4-en-3,17-dione was used as the substrate. It is interesting to note that DDQ is also capable of serving as an electron acceptor for the steroid 1-dehydrogenase which confirms the results of Ringold and Turner (16), who have shown that chemical 1,2-dehydrogenation using DDQ involves the abstraction of trans-diaxial hydrogens similar to that of the microbial system. The same enzyme system was capable of converting 9α-fluorohydrocortisone into 9α-fluoroprednisolone and the aromatization reaction exhibited an alkaline pH optimum around 9.0 which is characteristic for steroid 1-dehydrogenase. All these results support the view that the aromatization of 9α-hydroxyandrost-4-en-3,17-dione involved the introduction of a 1,2-double bond by the steroid 1-dehydrogenase.

TABLE IV.—EFFECT OF PHENAZINE METHOSULFATE ON METABOLISM OF 19-HYDROXYANDROST-4-EN-3,17-DIONE[a]

Additions	19-Hydroxyandrost-4-en-3,17-dione, μm. 20 min.	45 min.
None	1.57	1.44
NAD (1.5 μm.)	1.56	1.49
$NADH_2$ (1.2 μm.)	1.56	1.50
NADP (1.5 μm.)	1.59	1.56
$NADPH_2$ (1.2 μm.)	1.58	1.52
Phenazine methosulfate (1.0 μm.)	1.48	1.16

[a] The reaction mixture contained 1.65 μm. of 19-hydroxyandrost-4-en-3,17-dione in 0.2 ml. of dimethylformamide, and 40 units of enzyme (specific activity 900) in a total volume of 4 ml. of 0.03 *M* phosphate buffer, pH 7.0. The reaction was terminated by the addition of 1.0 ml. of chloroform at the indicated time intervals and the 19-hydroxyandrost-4-en-3,17-dione was estimated by the paper chromatographic method.

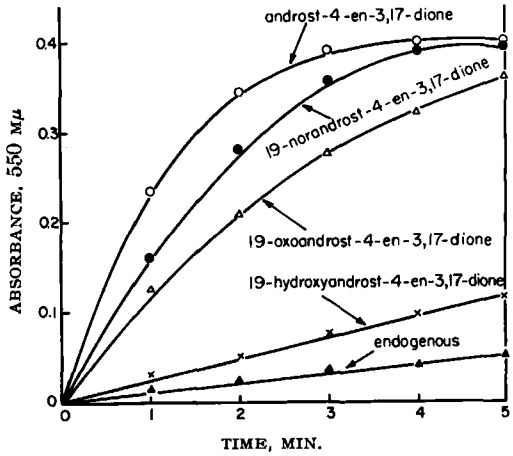


Fig. 4.—The relative rates of oxidation of 19-methyl, 19-nor, 19-oxo, and 19-hydroxy steroids by steroid 1-dehydrogenase. The assay system is the same as that of Fig. 3.

As the enzyme system failed to metabolize 9,10-secoandrost-4-en-3,9,17-trione, this obviously eliminated the alternate pathway B as depicted under Scheme II.

In the absence of an electron acceptor very little 9α-hydroxyandrost-4-en-3,17-dione was metabolized, and NAD and NADP showed no stimulation. Also, the kinetics of 9α-hydroxyandrost-4-en-3,17-dione oxidation (Fig. 3) showed no lag phase. All these results indicate that the first step in the metabolism of 9α-hydroxyandrost-4-en-3,17-dione involves a 1,2-dehydrogenation by the steroid 1-dehydrogenase. Although we have been unable to detect the presence of 9α-hydroxyandrosta-1,4-diene-3,17-dione, it is well known that vinylogs of β-hydroxyketones could undergo rearrangements very easily, and many attempts to prepare this type of compound have failed; *e.g.*, treatment of 19-acetoxyandrosta-1,4-diene-3,17-dione with $NaHCO_3$ or esterase resulted in the formation of estrone (17, 18).

19-Nor, 19-hydroxy, and 19-oxo androstenediones were converted into estrone when incubated with the same enzyme system. However, in the absence of an electron acceptor little 19-hydroxyandrost-4-en-3,17-dione was metabolized. This again eliminated an alternate mechanism involving deformylation prior to 1,2-dehydrogenation.

In view of the foregoing evidence, the aromatization of 9α-hydroxy and 19-hydroxyandrost-4-en-3,17-dione involve a direct 1,2-dehydrogenation to give vinylogs of β-hydroxyketones which undergo spontaneous nonenzymic rearrangements (reverse aldolization) to give their respective phenols. The microbial aromatizing system differs from that of the human placental microsomes in that (*a*) the relative rate of oxidation follows the order 19-nor>19-oxo>19-hydroxy, (*b*) oxygen and $NADPH_2$ are not required in the aromatization reaction, and (*c*) a suitable electron acceptor characteristic of flavoproteins is needed.

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Identification of Sympathomimetic Amines as Tetraphenylborates

By J. E. SINSHEIMER and EDWARD SMITH†

The tetraphenylborate salts of sympathomimetic amines are readily isolated in a highly pure state even from low concentrations in complex mixtures. Therefore, melting points, infrared, and ultraviolet spectral characteristics of these salts were studied as an aid to the identification of the medicinally important sympathomimetic amines.

CHATTEN AND LEVI (1) and Fischer and Plein (2) have prepared derivatives for the identification of sympathomimetic amines and have listed references for the identification of these amines. Characterization through tetraphenylborate (TPB) salts would be a valuable addition to these present methods for identifying sympathomimetic amines.

Isolation of organic bases as their TPB salts and subsequent identification by melting point is well established (3–12). Determination of various alkaloids and chemotherapeutic agents by ultraviolet spectra of their TPB salts has also been reported (13, 14). Chatten, Pernarowski, and Levi (15) have prepared the TPB salts of a series of local anesthetics and have reported their ultraviolet and infrared spectra.

In general, TPB derivatives of organic bases

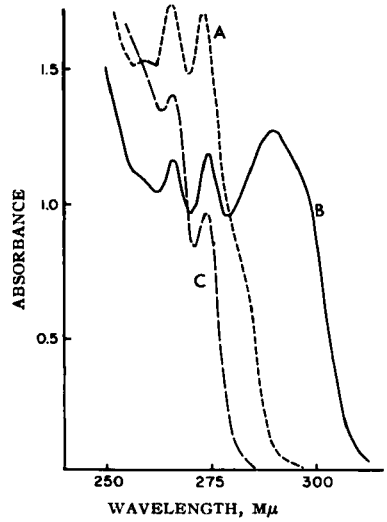

Fig. 1.—Ultraviolet spectra in methanol. Key: A, phenylephrine TPB ($2.53 \times 10^{-4}M$); B, methoxamine TPB ($6.46 \times 10^{-4}M$); C, ephedrine TPB ($4.83 \times 10^{-4}M$).

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Abstracted from a thesis presented by Edward Smith to the University of Michigan in partial fulfillment of Doctor of Philosophy degree requirements.
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In view of the foregoing evidence, the aromatization of 9α-hydroxy and 19-hydroxyandrost-4-en-3,17-dione involve a direct 1,2-dehydrogenation to give vinylogs of β-hydroxyketones which undergo spontaneous nonenzymic rearrangements (reverse aldolization) to give their respective phenols. The microbial aromatizing system differs from that of the human placental microsomes in that (*a*) the relative rate of oxidation follows the order 19-nor>19-oxo>19-hydroxy, (*b*) oxygen and $NADPH_2$ are not required in the aromatization reaction, and (*c*) a suitable electron acceptor characteristic of flavoproteins is needed.

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Identification of Sympathomimetic Amines as Tetraphenylborates

By J. E. SINSHEIMER and EDWARD SMITH†

The tetraphenylborate salts of sympathomimetic amines are readily isolated in a highly pure state even from low concentrations in complex mixtures. Therefore, melting points, infrared, and ultraviolet spectral characteristics of these salts were studied as an aid to the identification of the medicinally important sympathomimetic amines.

CHATTEN AND LEVI (1) and Fischer and Plein (2) have prepared derivatives for the identification of sympathomimetic amines and have listed references for the identification of these amines. Characterization through tetraphenylborate (TPB) salts would be a valuable addition to these present methods for identifying sympathomimetic amines.

Isolation of organic bases as their TPB salts and subsequent identification by melting point is well established (3–12). Determination of various alkaloids and chemotherapeutic agents by ultraviolet spectra of their TPB salts has also been reported (13, 14). Chatten, Pernarowski, and Levi (15) have prepared the TPB salts of a series of local anesthetics and have reported their ultraviolet and infrared spectra.

In general, TPB derivatives of organic bases

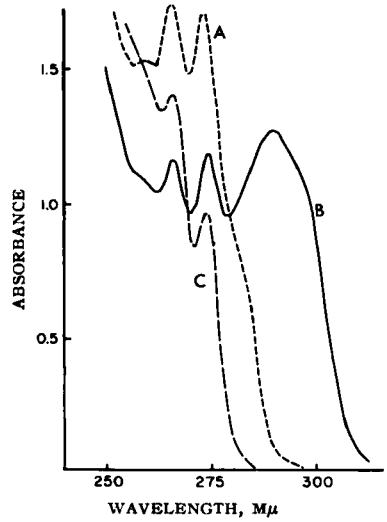

Fig. 1.—Ultraviolet spectra in methanol. Key: A, phenylephrine TPB ($2.53 \times 10^{-4}M$); B, methoxamine TPB ($6.46 \times 10^{-4}M$); C, ephedrine TPB ($4.83 \times 10^{-4}M$).

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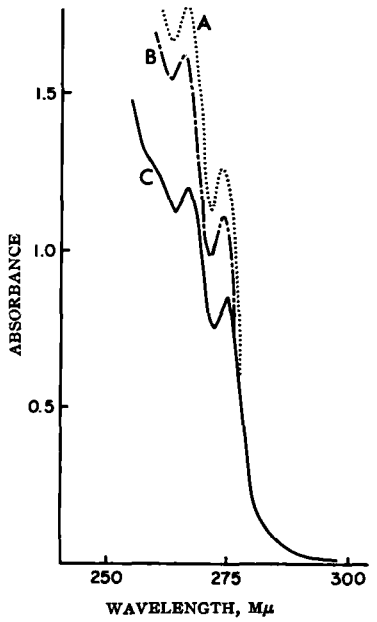

Fig. 2.—Ultraviolet spectra in methanol. Key: A, tetrahydrozoline TPB ($5.66 \times 10^{-4}M$); B, amphetamine TPB ($5.72 \times 10^{-4}M$): C, propylhexedrine TPB ($4.21 \times 10^{-4}M$).

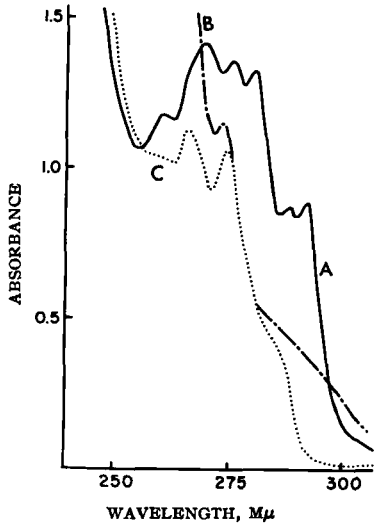

Fig. 3.—Ultraviolet spectra in methanol. Key: A, naphazoline TPB ($3.52 \times 10^{-4}M$); B, diethylpropion TPB ($5.07 \times 10^{-4}M$); C, hydroxyamphetamine TPB ($3.78 \times 10^{-4}M$).

are readily isolated in a pure form even from low concentrations in complex mixtures. Therefore, the melting points and spectral properties of these salts are recorded in this paper as an aid to the identification of this important group of amines.

EXPERIMENTAL

Apparatus and Materials.—Melting points were determined by the U.S.P. capillary tube method with a modified Thiele tube and a calibrated ASTM thermometer. Ultraviolet spectra were determined with the use of a Cary model 11 recording quartz spectrophotometer. Infrared spectral studies were made on Perkin-Elmer model 21 infrared spectrophotometers with sodium chloride or calcium fluoride optics.

Amine TPB salts were prepared as previously described (16) by addition of sodium TPB solution to acetate buffered solutions of the amine. The sympathomimetic amine salts studied and their melting points are listed in Table I. Reagents were of A.R. or U.S.P. grade and were used without additional purification.

The pH 4.6 acetate buffer used for preparation of

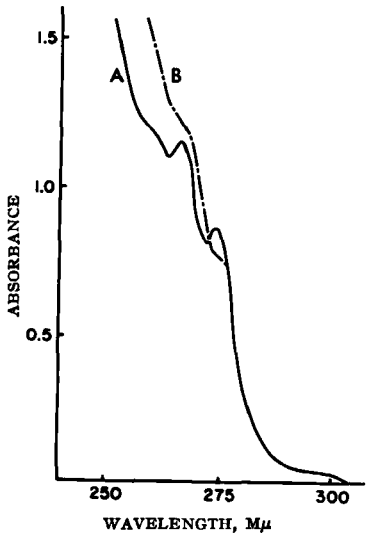

Fig. 4.—Effect of solvent change on sodium TPB spectra ($4 \times 10^{-4}M$). Key: A, methanol; B, acetate buffer.

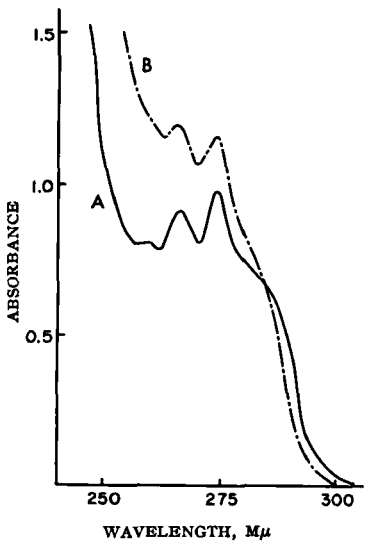

Fig. 5.—Effect of solvent change on epinephrine TPB spectra. Key: A, methanol ($2.35 \times 10^{-4}M$); B, acetate buffer ($2.87 \times 10^{-4}M$).

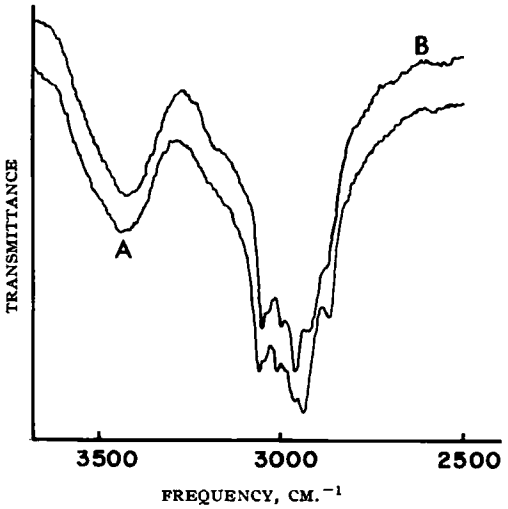

Fig. 6.—Infrared spectra with CaF_2 optics. Key: A, tuaminoheptane TPB; B, methylhexaneamine TPB.

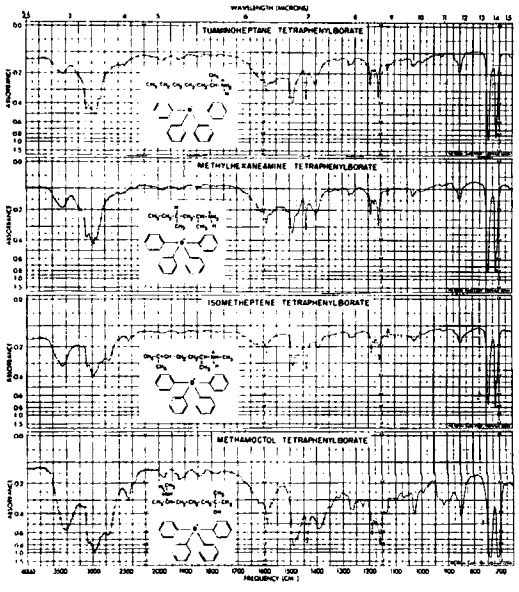

Fig. 7.—Infrared spectra.

the TPB salts and for the study of ultraviolet absorption spectra contained 0.8 mole each of acetic acid and sodium acetate per liter.

Determination of Spectra.—Ultraviolet absorption spectra were determined from 400 to 220 mμ in anhydrous methanol and in an acetate buffer. The infrared spectra were obtained from 4000 to

Table I.—Melting Points of Sympathomimetic Amine Salts

Compd.		M.p., °C. Tetraphenylborate		
		Compd.	Literature	Found
Amphetamine[a]	H_2SO_4	>300 dec.	114–116(10, 11)	114–116
Cyclopentamine[b]	HCl	113–115	...	149–151
Diethylpropion[c]	HCl	168–170	...	128–130 dec.
Ephedrine[n]	H_2SO_4	243–245 dec.	124(12) 135–138(5) 135 to 136.5(10)	128 to 129.5
Epinephrine[p]	...	211–212	...	135–138 dec.
Hydroxyamphetamine[a]	HBr	189–192	...	98–101
Isometheptene	Mucate	150–152	...	119.5 to 121
Isoproterenol[e]	H_2SO_4	127–129	...	119–120
Mephentermine[f]	H_2SO_4	215–220	...	158–159 dec.
Metaraminol[g]	Bitartrate	176–177	...	107–109
Methamoctol[d]	Mucate	157–158	...	106–108
Methamphetamine[a]	HCl	171–175	...	127–128
Methoxamine[h]	HCl	212–216	...	106–108
Methoxyphenamine[i]	HCl	124–128	...	132–134
Methylhexaneamine[b]	HCl	132–134	...	115–117
Naphazoline[j]	HCl	256–260	...	198–200
Nylidrin[k]	HCl	225–228 dec.	no precipitate	
Phenmetrazine[l]	HCl	176–178	...	149 to 150.5 dec.
Phenylephrine[q]	HCl	139–143	143 to 144.5(10)	141.5 to 142.5
Phenylethanolamine[r]	HCl	211–212	...	95 to 96.5
Phenylethylamine[o]	HCl	215–217	173–175(8)	173–175
Phenylpropanolamine[o]	HCl	195–196	...	119–120
β-Phenyl-*n*-propylamine[c]	HCl	123–124	...	157–159
Phenylpropylmethylamine[c]	HCl	144–148	...	128–129
Propylhexedrine[a]	HCl	127–128	...	143–145
Synephrine[s]	Tartrate	188–192	133.5 to 134(10)	133.4 to 134
Tetrahydrozoline[m]	HCl	255–257	...	200–202
Tuaminoheptane[b]	H_2SO_4	131–133	...	112–114
Tyramine[o]	HCl	266–269	129(4)	123 to 124.5

[a] Smith Kline and French Laboratories, Philadelphia, Pa. [b] Eli Lilly and Co., Indianapolis, Ind. [c] The Wm. S. Merrell Co., Cincinnati, Ohio. [d] Knoll Pharmaceutical Co., Orange, N. J. [e] Abbott Laboratories, North Chicago, Ill. [f] Wyeth Laboratories, Philadelphia, Pa. [g] Merck Sharp and Dohme Laboratories, West Point, Pa. [h] Burroughs Wellcome Co., Tuckahoe, N. Y. [i] The Upjohn Co., Kalamazoo, Mich. [j] Ciba Pharmaceutical Products, Inc., Summit, N. J. [k] U. S. Vitamin and Pharmaceutical Corp., New York, N. Y. [l] Geigy Pharmaceuticals, Yonkers, N. Y. [m] Chas. Pfizer and Co., Inc., Brooklyn, N. Y. Grateful acknowledgement is made for the compounds supplied by these manufacturers. The balance of the compounds were purchased as follows: [n] Merck and Co., Inc., Rahway, N. J. [o] Eastman Kodak, Rochester, N. Y. [p] Matheson, Coleman and Bell, East Rutherford, N. J. [q] Winthrop Laboratories, New York, N. Y. [r] K & K Laboratories, Jamaica, N. Y. [s] L. Light and Co., Colnbrook, England.

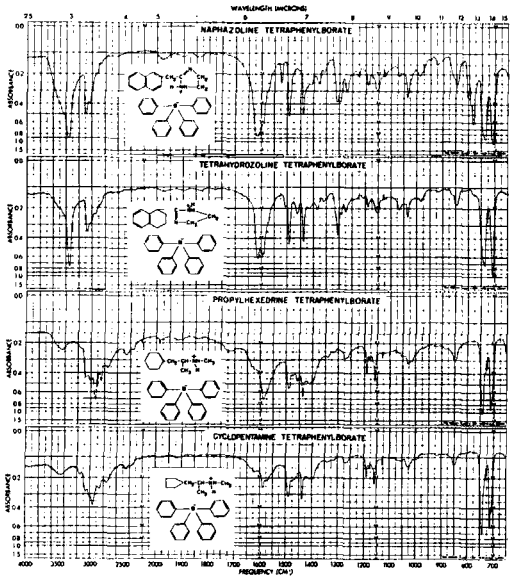

Fig. 8.—Infrared spectra.

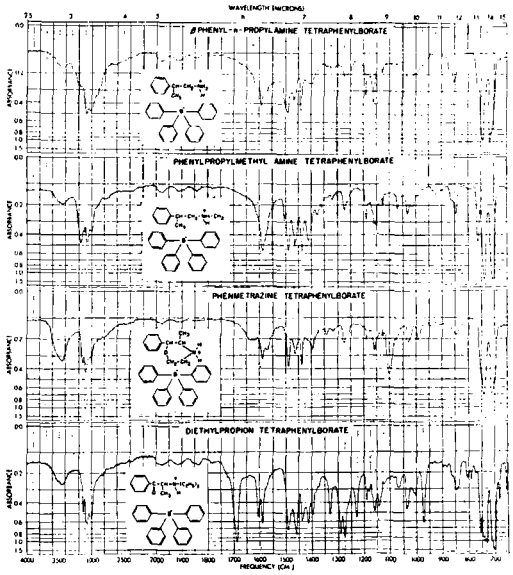

Fig. 10.—Infrared spectra.

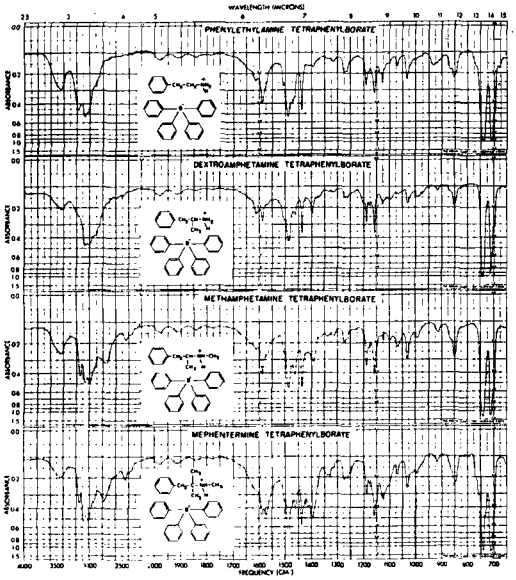

Fig. 9.—Infrared spectra.

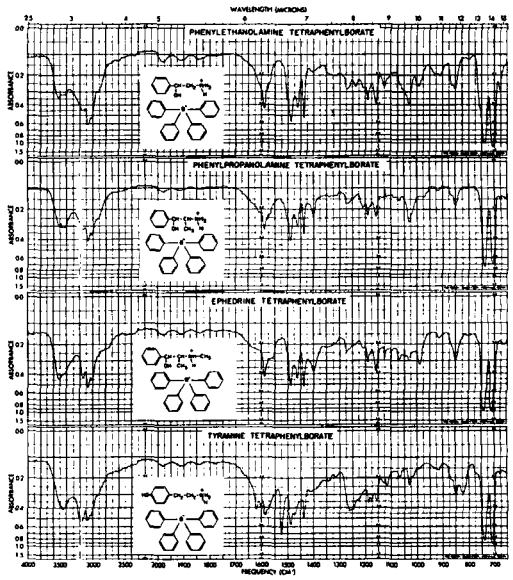

Fig. 11.—Infrared spectra.

650 cm.$^{-1}$ with the use of KBr disks. Approximately 1-mg. samples of dried TPB salts were mixed with 200 mg. of potassium bromide for 30 seconds with a steel capsule and pestle on a Wig-L-Bug.[1] The mixed sample in a KBr die was subjected to vacuum for 5 minutes before and also while being pressed at 20,000 p.s.i. for 5 minutes.

DISCUSSION

The rate of formation of the TPB salts was the same as in the amperometric titration of these amines with TPB (16). Precipitates formed rapidly, except for phenol and catechol derivatives. Phenol derivatives generally precipitated after 1 hour, except with metaraminol and nylidrin. No TPB salt could be obtained from nylidrin, while TPB salts of metaraminol and catechol derivatives could only be prepared after stirring for several hours.

Washing TPB salts free of excess reagent and drying under vacuum yields compounds which are sufficiently pure for identification purposes (8, 9, 15). Crane has found crystallization to be unnecessary and to decompose TPB salts in some instances (8).

The melting or decomposition points observed are listed in Table I.

[1] Crescent Dental Manufacturing Co.

Ultraviolet Spectra. —The ultraviolet spectra of amine TPB salts are summations of the absorbance of TPB and parent amine. Therefore, any strong absorbance maxima of the amine can still be detected; characterization of the U.V. spectra of sympathomimetic amine TPB salts follows that of the spectra of the parent amine, *per se*, as reported for example by Thies and Özbilici (17). Phenylalkyl, phenolic, and catechol amine derivatives can be distinguished by changes in absorbance in the region of the TPB maxima (266 and 274 mμ). Typical spectra of TPB salts are given in Figs. 1-3. Figure 4 illustrates the contribution due to TPB. Table II is a summary of molar absorptivities of TPB salts of the amines and includes sodium TPB for comparison.

The ultraviolet characteristics of most TPB salts in acetate buffer are similar to those in methanol. The disappearance of the minima of the TPB absorbance (264 and 272 mμ) is the predominant change and results in the appearance of shoulders instead of peaks as illustrated in Fig. 4. Phenol derivatives as illustrated in Fig. 5 represent an exception in that minima were observed and the relative intensity of the peaks was altered by a change to the acidic media.

Infrared Measurements.—The TPB ion exhibits absorbance characteristics due to monosubstituted phenyl and boron-aryl (18, 19) groups as shown by Chatten, Pernarowski, and Levi (15) for sodium TPB. It is noted that potassium bromide itself readily absorbs moisture, producing additional absorbance in the 3500 and 1650 cm.$^{-1}$ regions. Although there is a similarity in spectra because of the absorbance due to the TPB group, no two spectra are identical.

The differentiation of closely related compounds can be illustrated by a comparison of the spectra of tuaminoheptane and methylhexaneamine tetraphenylborates. As shown in Fig. 6, calcium fluoride optics accentuate spectral differences for the two compounds in the CH stretching region, a difference which is difficult to detect with sodium chloride optics as illustrated by the spectra of these two salts in Fig. 7. Methylhexaneamine has a definite increase in the CH_3 stretching absorbance at 2962

TABLE II.—ULTRAVIOLET MOLAR ABSORPTIVITIES OF TETRAPHENYLBORATES IN METHANOL

Compd.	Molar Absorptivity at 266 mμ[a]	Molar Absorptivity at 274 mμ[a]
PHENYLALKYLAMINES		
Amphetamine	2710	1860
Diethylpropion	...	2260
Mephentermine	2800	1940
Methamphetamine	2960	2020
Phenmetrazine	2900	1970
Phenylethylamine	2710	1850
β-Phenyl-*n*-propylamine	2690	1850
Phenylpropylmethylamine	2810	1930
BENZYLIC HYDROXYL AMINES		
Ephedrine	2920	2010
Phenylethanolamine	2770	1910
Phenylpropanolamine	2820	1940
PHENOLIC AMINES		
Hydroxyamphetamine	2970	2800
Metaraminol	3820	3670
Phenylephrine	3440	3360
Synephrine	3380	3080
Tyramine	3590	3400
CATECHOLAMINES		
Epinephrine	3910	4180 (274.5 mμ)
Isoproterenol	3910	4250 (275 mμ)
METHOXYPHENYLALKYLAMINES		
Methoxamine	2730	2770 (274.5 mμ) 2790 (291 mμ)
Methoxphenamine	2600	2400 (273.5 mμ)
ALIPHATIC AMINES		
Cyclopentamine	2790	1960
Isometheptene	2850	2000
Methamoctol	2710	1900
Methylhexaneamine	2780	1970
Propylhexedrine	2860	2040
Tuaminoheptane	2690	1890
IMIDAZOLES		
Naphazoline	2490 (291 mμ) 3740 (280 mμ) 4000 (268 mμ)	156 (312 mμ) 2460 (288 mμ) 3830 (275.5 mμ) 3324 (261 mμ)
Tetrahydrozoline	3150	2250
SODIUM	2980	2070

[a] Exception to the 266 mμ and 274 mμ maxima are given in parenthesis; absorptivities then correspond to these different maxima.

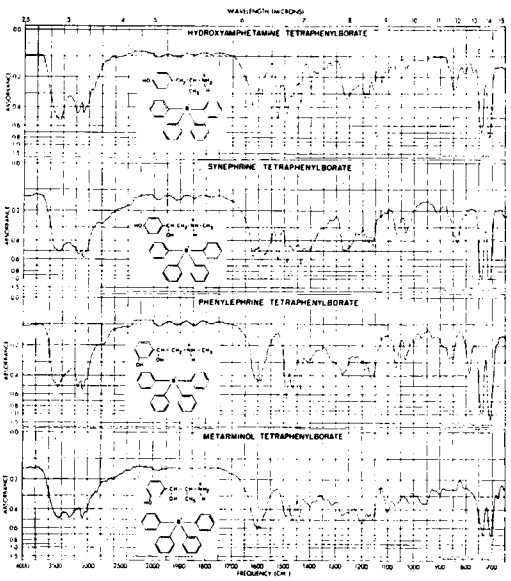

Fig. 12.—Infrared spectra.

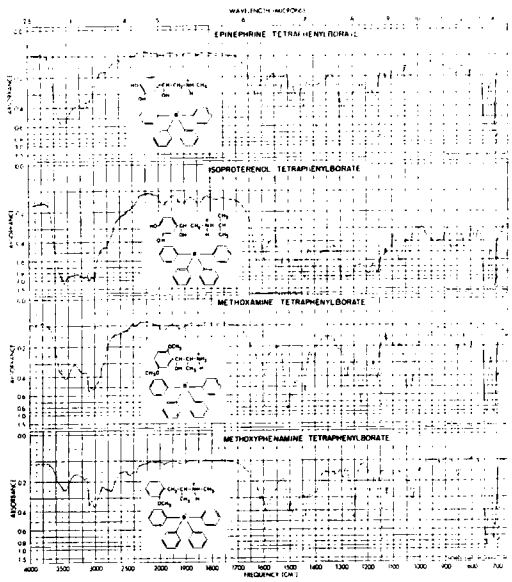

Fig. 13.—Infrared spectra.

and 2872 cm.$^{-1}$ compared to the CH_2 stretching absorbance at 2926 and 2853 cm.$^{-1}$ This is consistent with the increased CH_3 to CH_2 ratio from 2:4 in tuaminoheptane to 3:2 in methylhexaneamine.

The spectra of sympathomimetic amine TPB salts obtained with potassium bromide pellets on the model 21 spectrophotometer with sodium chloride optics are illustrated in Figs. 7–13. It can be seen from these spectra that TPB salts serve as a convenient medium for the isolation and identification of various members of a series of amines.

SUMMARY

The preparation of TPB salts of sympathomimetic amines together with their melting points, ultraviolet, and infrared spectra have been shown to be useful for the identification of these amines.

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Advantages of the Hematocrit Method for Testing Isotonicity of Injectable Solutions

By IVO SETNIKAR and OLIMPIA TEMELCOU

Fifty solutions of substances of pharmaceutical interest, all found nonhemolytic at iso-osmotic concentration by Hammarlund and Pedersen-Bjergaard (11) on human blood, were tested for their hemolytic properties and isotonicity on rabbit red cells using the hematocrit method. It was demonstrated that several of these solutions showed some hemolytic properties on rabbit red cells because they were not isotonic at iso-osmotic concentration. Other solutions precipitated hemoglobin; this explains the absence of hemolysis when the hemolytic method was used. The reasons are given for preferring the hematocrit method to the hemolytic method for testing isotonicity and general compatibility with blood.

THE HEMOLYTIC METHOD (1–12) is probably among the simplest for testing the compatibility of injectable solutions with erythrocytes. However, it has the following drawbacks when the isotonicity of solutions has to be tested.

(*a*) It is not possible to calculate the isotonic concentration from a hemolysis-preventing concentration because the ratio between these two concentrations can vary considerably (14, 15). (*b*) The compatibility is tested by adding a very small quantity of blood to the solution. Thus the environment is very different from the actual conditions of injection. In particular, the pH depends on the pH of the solution and is not buffered by the blood (11). As a result, the information on the compatibility of the solution with erythrocytes may be misleading. (*c*) In absence of hemolysis it is difficult to distinguish between a real prevention of hemolysis and a precipitant action on hemoglobin (9, 15, 16). (*d*) The substances present in the solution may alter the colorimetric determination of hemoglobin in solution either by altering the absorbance of hemoglobin or by interfering with their own color with the colorimetric reading (11). For this reason the quantitative appreciation of hemolysis may be incorrect.

The hematocrit method (15, 16), which gives direct information on the isotonicity of a solution and which is performed in environmental conditions much closer to the actual conditions of injection, therefore seems better suited to test injectable solutions for isotonicty with erythrocytes and for general compatibility with blood.

In this paper a series of substances were tested for their isotonicity on rabbit red cells using the hematocrit method. All these substances were selected from those which, at iso-osmotic concentrations, were found not hemolytic on human blood by Hammarlund and Pedersen-Bjergaard (11).

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METHOD

The solutions to be tested were mixed 1 : 1 (v/v) with a suspension of red cells as previously described (16).

The hematocrit determinations were performed using tubes of 3-mm. diam. and a length of 100 mm. in duplicate on serial concentrations of the solution to be tested. In the concentration scale, the iso-osmotic and (if possible) the isotonic concentration, were included. Hemolysis on the supernatant was classified in the following way: absent (0); light (L), with a concentration of hemoglobin up to 0.5%; medium (M), with a hemoglobin concentration from 0.5 to 3%; heavy (H), with a hemoglobin concentration of more than 3%. The hemoglobin concentration was not determined on the supernatant of each sample but by comparing the sample with hematocrit tubes containing hemoglobin solutions in serial concentration. Complete hemolysis gave a hemoglobin concentration of 15%.

RESULTS

Rabbit Red Cells.—The substances tested for isotonicity and for hemolytic effects by the hematocrit method are listed in Table I. The iso-osmotic concentrations given in Table I are based on the experimental data obtained with human erythrocytes by Hammarlund and Pedersen-Bjergaard (17).

With regard to hemolysis, Table I shows that the results obtained on the carbohydrates and polyalcohols, all with six or more C atoms, were in agreement with those found by the hemolytic method by other investigators (8, 11). Moreover, the isotonic concentration found for these compounds was equal to the iso-osmotic concentration.

Iso-osmotic concentrations of calcium, magnesium, potassium, and sodium salts provoked in many instances a light or moderate hemolysis in disagreement with the results obtained by the hemolytic method. For 13 out of 32 of these solutions, the hematocrit method showed that the isotonic concentration for rabbit blood was different from the iso-osmotic concentration.

The miscellaneous substances, at iso-osmotic concentration, produced hemolysis with the exception of tetraethylammonium chloride. It was found also that the isotonic concentrations were

consistently higher than the iso-osmotic concentration.

Some of the solutions described in Table I under *Miscellaneous* were tested also by the hemolytic method on rabbit blood following the procedure described by Shaw and Husa (13). The compatibility of these solutions with rabbit hemoglobin was tested, adding to 10 ml. of these solutions 0.1 ml.

TABLE I.—ISOTONIC CONCENTRATIONS AND HEMOLYSIS OBSERVED WITH THE HEMATOCRIT METHOD ON RABBIT RED CELLS

	Iso-osmotic Concn., %	Isotonic Concn. Range, %	Hemolysis Observed at Iso-osmotic Concn.	Hemolysis Observed at Isotonic Concn.	Approximate Ratio between Isotonic and Iso-osmotic Concn.
Carbohydrates and Polyalcohols					
Dextrose U.S.P.	5.51	5.5 to 5.6	O	O	1
d-Fructose	5.05	5.0 to 5.1	O	O	1
Lactose U.S.P.	9.75	9.5 to 10.0	O	O	1
Mannitol U.S.P.	5.07	5.0 to 5.1	O	O	1
Sorbitol $^1/_2$ H_2O	5.48	5.4 to 5.5	O	O	1
Sucrose U.S.P.	9.25	9.0 to 9.5	O	O	1
Calcium and Magnesium Salts					
Calcium chloride·6 H_2O	2.50	2.4 to 2.5	O	O	1
Calcium lactate N.F.	4.50	4.4 to 4.6	O	O	1
Calcium pantothenate U.S.P.	5.50	5.4 to 5.6	O	O	1
Magnesium chloride	2.02	2.0 to 2.1	O	O	1
Magnesium sulfate·7 H_2O	6.30	5.0 to 5.1	O	O	0.8
Potassium Salts					
Chlorate N.F.	1.88	1.8 to 2.0	L	L	1
Chloride U.S.P.	1.19	1.1 to 1.2	O	O	1
Iodide U.S.P.	2.59	2.5 to 2.6	L	L	1
Nitrate N.F.	1.62	1.6 to 1.7	L	L	1
Penicillin G U.S.P.	5.48	9.5 to 10.0	H	M	1.8
Phosphate monobasic	2.18	2.7 to 2.8	O	O	1.3
Sulfate	2.11	1.7 to 1.8	L	O	0.8
Sodium Salts					
Acetate anhydrous	1.18	1.6 to 1.8	L	O	1.4
Ascorbate	3.00	3.0 to 3.1	O	O	1
Barbital	3.12	2.9 to 3.0	O	O	0.9
Benzoate U.S.P.	2.25	2.3 to 2.6	M	M	1
Bicarbonate	1.39	1.3 to 1.4	O	O	1
Biphosphate·2 H_2O	2.77	2.8 to 2.9	O	O	1
Bisulfite U.S.P.	1.50	2.0 to 2.1	O	O	1.4
Borate U.S.P.	2.60	2.6 to 2.7	M	M	1
Cacodylate N.F.	3.30	3.0 to 3.1	O	O	0.9
Chloride	0.90	0.91 to 0.93	O	O	1
Citrate U.S.P.	3.02	2.3 to 2.4	O	O	0.7
Fluorescein U.S.P.	3.34	4.5 to 4.6	?	?	1.3[a]
Iodide U.S.P.	2.37	2.2 to 2.3	M	M	1[b]
Nitrate	1.36	1.0 to 1.1	L	L	0.8[c]
Nitrite U.S.P.	1.08	1.0 to 1.1	O	O	1[d]
Phosphate dibasic·2 H_2O	2.23	1.6 to 1.7	O	O	0.7
Propionate N.F.	1.47	1.4 to 1.5	O	O	1
Salicylate U.S.P.	2.53	4.0 to 4.1	M	M	1.6
Sulfate anhydrous	1.61	1.5 to 1.6	O	O	1
Thiosulfate N.F.	2.98	2.6 to 2.7	O	O	0.9
Miscellaneous					
Ammonium phosphate dibasic	1.76	3.1 to 3.3	H	O	1.8[e]
Ammonium sulfate	1.68	5.0 to 5.2	H	L	3[f]
Amphetamine phosphate N.F.	3.47	>16	H	?	>5[f]
Amphetamine sulfate U.S.P.	4.23	9.0 to 9.2	M	H	2
Atropine sulfate U.S.P.	8.85	>15	L	?	>1.7[g]
Decamethonium bromide	5.00	6.2 to 6.4	H	H	1.3
Dextro-amphetamine sulfate U.S.P.	4.20	10.0 to 10.5	H	H	2.4[h]
Ephedrine sulfate U.S.P.	4.54	13.0 to 13.5	L	H	3
Hexamethonium chloride	3.30	...	...	...	[i]
Phenol U.S.P.	2.80	...	...	...	[j]
Silver Nitrate U.S.P.	2.74	...	...	...	[i]
Tetramethylammonium chloride	2.67	3.4 to 3.5	O	O	1.3

[a] Hemolysis not appreciable because of the intense coloration of the sodium fluorescein solution. [b] An increase of concentration increases hemolysis and diminishes hematocrit values. [c] Denaturation and flocculation of hemoglobin. [d] Dark color of the red cell column. [e] Transparent red cell column. [f] Transparent red cell column with some floccules. [g] Transparent and brownish-red cell column. [h] The minimum hemolysis was observed at concentrations ranging from 6 to 8%. [i] Dark precipitate. [j] Precipitation.

TABLE II.—HEMOLYTIC AND HEMOGLOBIN PRECIPITATING ACTIVITIES OF SOME SOLUTIONS

	Iso-osmotic Concn., %	Hemolytic Method		Compatibility with Rabbit Hemoglobin	
		Hemolysis	Color Changes	Precipitation	Color Changes
Ammonium phosphate dibasic	1.76	O[a]		...	...
Ammonium sulfate	1.68	L[a]		...	...
Amphetamine phosphate N.F.	3.47	O[a]		...	...
Amphetamine sulfate U.S.P.	4.23	L[a]		...	...
Atropine sulfate U.S.P.	8.85	O[a]	dark	...	dark
Phenol U.S.P.	2.80	O	grey	complete	grey
Silver nitrate U.S.P.	2.74	O	dark	almost complete	dark

[a] A layer of hemoglobin in solution was noted on the surface of the red cell sediment.

of a 20% rabbit hemoglobin solution obtained from red cells washed three times with 0.9% NaCl and hemolyzed with repetitive freezing.

Table II shows that the results obtained on ammonium phosphate and sulfate, amphetamine phosphate and sulfate, and atropine sulfate roughly agree, in regard to hemolysis, with those described by Hammarlund and Pedersen-Bjergaard (11). However, a thin layer of hemoglobin in solution was always noted above the red cell sediment, showing that a partial hemolysis was present and explaining the hemolysis observed with the hematocrit method (*cf.* Table I). The hematocrit method seems therefore more sensitive for hemolysis, probably because comparatively about 100 times more red cells are added to the solution to be tested.

Phenol and silver nitrate provoked precipitation of hemoglobin; this explains the absence of hemolysis reported by Hammarlund and Pedersen-Bjergaard (11). The precipitation appeared very clearly in the hematocrit tubes showing the incompatibility of these substances with blood. Using the hemolytic method, a darkening and shrinking of the red cells with a milky appearance of the solution (for phenol) was reported (11)—phenomena which may be sometimes overlooked and not necessarily attributed to an incompatibility with blood.

Hemolysis—as shown in Table I for amphetamine sulfate, dextro-amphetamine sulfate, and ephedrine sulfate—is not necessarily correlated with osmotic pressure, since on increasing the concentration of these substances an increase of the osmotic pressure can be demonstrated together with an increase of hemolysis. An example of this behavior is given in Table III.

Human Red Cells.—Since Hammarlund and Pedersen-Bjergaard obtained their results on human red cells and since a difference between human and rabbit red cells is demonstrated in osmotic behavior (3, 15, 16), the hematocrit method was performed for some solutions on human red cells to establish whether the disagreement found between the results obtained by the hematocrit and the hemolytic method might be attributed to species differences.

TABLE III.—HEMATOCRIT VALUE AND HEMOLYSIS OBSERVED ADDING ONE VOLUME OF RABBIT RED CELLS TO ONE VOLUME OF DEXTRO-AMPHETAMINE SULFATE IN DIFFERENT CONCENTRATIONS

Dextro-amphetamine Sulfate Concn., %	Hematocrit Value	Hemoglobin in the Supernatant, %	Estimated Percentage of Hemolyzed Cells
4.0	0.87	5.5	4.3
5.0	0.76	2.2	3.5
6.0	0.67	0.3	0.7
7.0	0.63	0.2	0.4
8.0	0.56	0.3	0.9
9.0	0.54	2.5	7.3
10.0	0.50	5.1	17.0
Plasma	0.50	0	0

TABLE IV.—HEMATOCRIT VALUES AND HEMOLYSIS OBSERVED ON HUMAN RED CELLS WITH ISO-OSMOTIC CONCENTRATIONS OF DIFFERENT SUBSTANCES

Substance	Iso-osmotic Concn., %	Hematocrit Value	Hemolysis
Amphetamine sulfate U.S.P.	4.23		total
Atropine sulfate U.S.P.	8.85	0.65	light
Decamethonium bromide	5.00		total
Dextro-amphetamine sulfate U.S.P.	4.20	0.80	high
Ephedrine sulfate U.S.P.	4.54	0.75	high
Sodium acetate anhydrous	1.18	0.50	0
Sodium benzoate U.S.P.	2.25	0.50	light
Sodium citrate U.S.P.	3.02	0.48	0
Sodium phosphate dibasic $\cdot 2H_2O$	2.23	0.50	0
Plasma	..	0.50	0

In Table IV a hematocrit value higher than 0.50 indicates hypotonicity—a hematocrit value lower than 0.50, hypertonicity. Table IV shows that the results obtained on human red cells reproduce those obtained on rabbit red cells, with very slight differences for sodium acetate and dibasic sodium phosphate, the first of which showed a little higher osmotic pressure on human red cells and the second a little higher osmotic pressure on rabbit red cells compared to the red cells of the other species.

DISCUSSION

The results presented in this paper and those of the literature (1, 16) show that relatively few molecules or ions are unable to penetrate the red cells and therefore (18) to exert an osmotic pressure through the cell membrane. For this reason it must again be emphasized that isotonicity can be tested only by putting the solutions directly in contact with cells and cannot be calculated on the basis of the molecular weight and probable micellar behavior, or on the basis of physical methods as is suggested

also in recent literature (20). The danger involved in these procedures was demonstrated for iso-osmotic urea solutions by Setnikar and Temelcou (16).

Using the hematocrit method it can be demonstrated that, among the substances which are of interest for pharmaceutical formulation, some carbohydrates and polyalcohols exert an osmotic pressure through the erythrocyte membrane which can be predicted on the basis of the colligative physical properties of these solutions. For these solutes the red cell membrane appears therefore impermeable.

The same can be said for salts with calcium, magnesium, potassium, and sodium cations. In these cases the osmotic pressure exerted through the cell membrane is the same as if the total amount of ions in solutions was prevented from passing through the cell membrane. This confirms the view that it is enough for the membrane to be impermeable to the cation or to the anion component of a salt to be impermeable to the whole salt of which the ion is a part (19).

Particular mention should be given to sulfate and phosphate ions, which, according to Davson (19), impede the penetration of the cation component into the red cells so that the whole salt can exert an osmotic pressure. The results of this paper show, however, that the iso-osmotic concentration of the phosphate and sulfate salts (*cf.* the ammonium, amphetamine, atropine, and ephedrine salts reported in Table I) is always lower than the isotonic concentration, that iso-osmotic concentrations always produce partial hemolysis and that often these substances affect the red cells in a way which makes the red cell column almost transparent. Therefore, these anions can not be considered equal (as Hammarlund and Pedersen-Bjergaard (11) suggested) to the cations already mentioned, which are unable to cross the cell membrane.

Among the really impermeable ions tetraethylammonium must finally be mentioned (*cf.* Table I).

Norlander and Sandell (21) and Setnikar and Paterlini (22) pointed out that for hypodermic use, strict isotonicity is not critical for good tolerance. The same was demonstrated by Hammarlund and Pedersen-Bjergaard (11) for intravenous solutions when small quantities are injected. This seems to diminish the importance of an accurate method as the hematocrit one for testing isotonicity of injectable solutions. Such a method becomes, however, indispensable for solutions which must be injected in large quantities into the blood stream or in some particular cases, *e.g.*, for solutions designed for rachidean administration. In this case the small quantity of cerebrospinal fluid and its slow replacement (23) can not buffer liquids which are not isotonic to a tolerable osmotic pressure, so that the very delicate nervous cells may be damaged.

In any case, it is very important to recognize damaging solutions, *i.e.*, strongly hemolytic (of the saponin type) or with a precipitating or denaturing action on proteins (as zinc sulfate, phenol, etc.). The former are easily detected both with the hemolytic and the hematocrit method; the latter are more immediately detected with the hematocrit method because a precipitation on the red cell column is immediately noted.

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Qualitative and Quantitative Tests for Stannous Fluoride

By JOHN J. HEFFERREN

A series of qualitative and quantitative assay procedures are presented for stannous fluoride. These data are the result of a cooperative project of the American Dental Association with the major manufacturers and users of stannous fluoride. The physical and chemical characteristics of stannous fluoride are described. The methods selected to define stannous fluoride and other methods which might be utilized are discussed.

STANNOUS FLUORIDE is an agent which has been used topically as a simple solution, in dentifrices, and in other mixtures to reduce the incidence of dental caries (1). This monograph is part of a continuing program to provide data on those drugs and chemicals which are described in "Accepted Dental Remedies." The tests are designed to be applied to stannous fluoride as such and to capsules and aqueous solutions containing stannous fluoride. The limits set in these tests are based on normal analytical and manufacturing variation.

It should be recognized that the particular tests and methods described do not necessarily represent those used by any particular firm, but rather those which have been selected as generally applicable. It is expected that there will be capsules or solutions of stannous fluoride containing interfering substances which will require modification of these methods. The terminology of the "United States Pharmacopeia" has been used wherever possible.

DESCRIPTION

Physical Properties.—Stannous fluoride (SnF_2; mol. wt. 156.70) is a white, crystalline solid with a bitter, salty taste, m.p. 212–214°, b.p. 845–855°. It is freely soluble in water and practically insoluble in alcohol, ether, and chloroform. The pH of an 8% aqueous solution is about 2.3.

Identity Tests.—Transfer to a small test tube about 100 mg. of stannous fluoride and dissolve in 10 ml. of water. Mix on a spot plate 2 drops of this solution with 2 drops of 1% silver nitrate; a brown-black precipitate forms (presence of stannous ion).

Transfer to a small test tube 2 drops of mercuric chloride T.S. (6.5 Gm./100 ml. water) and add 1 drop of the above stannous fluoride solution; a white milky precipitate forms. Further addition of stannous fluoride solution gives a brown-black precipitate (presence of stannous ion).

Transfer 1 drop of the above stannous fluoride solution to a spot plate, add 1 drop of 0.001 *M* alizarin complexan and 1 drop of 0.001 *M* cerous nitrate; a lilac-blue color forms (presence of fluoride ion).

Purity Tests.—Dry about 1 Gm. of stannous fluoride, accurately weighed, at 105° for 4 hours; the loss in weight is not more than 0.5%.

Transfer 10 Gm. of stannous fluoride, accurately weighed, to a 400-ml. plastic beaker and add 200 ml. of water. Stir with a plastic rod for 3 minutes or until no more solid dissolves. Filter the mixture through a tared Gooch crucible tightly packed with asbestos fiber. Thoroughly wash first with about 50 ml. of 1% ammonium fluoride and then with about 200 ml. of water. Dry the residue at 105° for 4 hours and weigh. The water-insoluble residue does not exceed 0.2%.

ASSAY

Stannous Ion.—Place 200 ml. of water, 100 ml. of hydrochloric acid, and a few boiling chips in a 500-ml. Erlenmeyer flask. Boil the mixture for 10 minutes; add 0.25 Gm. of stannous fluoride, accurately weighed. In an inert atmosphere such as carbon dioxide or oxygen-free nitrogen, swirl the flask to dissolve the stannous fluoride, and cool the solution to room temperature. Add 5 ml. of starch T.S. and titrate in an inert atmosphere with 0.1 *N* potassium iodate to the blue starch end point. Each milliliter of 0.1 *N* potassium iodate is equivalent to 7.835 mg. of stannous fluoride. The amount of stannous fluoride, SnF_2, is not less than 95.0% nor more than 105.0%.

Preparation of Potassium Iodate Titrant.—Transfer about 3.6 Gm. of potassium iodate in 200 ml. of oxygen-free water containing 1 Gm. of sodium hy-

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droxide and 10 Gm. of potassium iodide to a 1000-ml. volumetric flask; dilute with oxygen-free water to the mark and mix.

Standardization of Potassium Iodate Titrant.—Transfer 0.25 Gm. of reagent grade tin, accurately weighed, to a 500-ml. Erlenmeyer flask, add 100 ml. of hydrochloric acid, and 2 drops of 2% antimony trichloride, and allow the mixture to stand at room temperature until all the tin has dissolved. Carefully add 180 ml. of water and 10 ml. of sulfuric acid and mix. Add a nickel coil, prepared from a coiled piece of 3 × 6-in. reagent grade nickel sheet which has been washed with petroleum ether, boiled in 1:2 hydrochloric acid for 5 minutes and rinsed with water. (The coil should be treated with acid before each use.) Add 5 Gm. of hydrogen-reduced iron powder, and wash down the sides of the flask with water. Gently boil the mixture for 20 minutes in an inert atmosphere. Maintaining the inert atmosphere, cool the mixture to room temperature, add 5 ml. of starch T.S., and titrate with the potassium iodate titrant to the blue starch end point. Each milliliter of 0.1 *N* potassium iodate is equivalent to 5.935 mg. of tin.

Soluble Tin.—Transfer 0.1 Gm. of stannous fluoride, accurately weighed, to a 500-ml. volumetric flask. Add about 300 ml. of water, vigorously shake for about 5 minutes, fill to the mark with water, and mix. Filter a portion of the mixture through a piece of hard filter paper, discarding the first 10 ml. of filtrate. Transfer 2 ml. of the filtrate to a 50-ml. volumetric flask and add 10 ml. of 30% sulfuric acid and 5 drops of thioglycolic acid. Similarly, prepare a blank and three standard tin solutions containing 2, 5, and 10 ml., respectively, of *Standard Tin Solution B*, which contains 0.04 mg. tin/ml. Dilute each of these solutions with water to a total volume of 40 ml. Add 2 ml. of 2% sodium lauryl sulfate and gently swirl to mix with a minimum of foaming. With gentle swirling, add 2 ml. of *dithiol solution*, cool to room temperature, fill to the mark with water, and mix. Spectrophotometrically determine the absorbances of the solutions containing tin in 1-cm. cells at the maximum at about 530 mμ using the reagent blank as a reference. Prepare a plot of absorbance *versus* tin concentration using the absorbances of the standard tin solutions. Determine the tin concentration in the stannous fluoride solutions using this plot. The amount of stannous fluoride is not less than 95.0% nor more than 105.0%.

Preparation of Reagent.—Standard Tin Solution A.—Transfer 0.2 Gm. of reagent grade tin, accurately weighed, to a 1000-ml. volumetric flask and dissolve in 250 ml. of hydrochloric acid. Cool the solution to room temperature, fill to the mark, and mix. This solution is stable for about 1 month.

Standard Tin Solution B.—Transfer 50 ml. of *Standard Tin Solution A* to a 250-ml. volumetric flask, fill to the mark with water, and mix. This solution should be freshly prepared.

Dithiol Solution.—Transfer 0.15 Gm. of dithiol to a 100-ml. beaker, add 8 drops of thioglycolic acid, and 50 ml. of 2% sodium hydroxide, and stir to dissolve the dithiol. Filter the solution if it is not clear. The dithiol solution stored in a glass-stoppered bottle in the refrigerator is stable for about 1 week. The solution should be discarded if a precipitate forms.

Soluble Fluoride.—Transfer 0.2 Gm. of stannous fluoride, accurately weighed, to a 100-ml. volumetric flask. Add about 70 ml. of water, vigorously mix for about 5 minutes, fill to the mark with water, and mix. Transfer 10 ml. of this solution to a 100-ml. volumetric flask, fill to the mark with water, and mix. Transfer 10-ml. of the last solution to a third 100-ml. volumetric flask, fill to the mark with water, and mix. Prepare a standard fluoride solution containing 10 mcg./ml. of fluoride ion using sodium fluoride reagent. Transfer 1, 2, 3, and 4 ml. of the standard fluoride solution and 5 ml. of the final stannous fluoride solution to 100-ml. volumetric flasks. Add 10 ml. of zirconium-SPADNS reagent to each of the above volumetric flasks plus a flask for the blank, fill to the mark with water, and mix. Spectrophotometrically determine the absorbances of the blank and fluoride-containing solutions in 1-cm. cells at the maximum at about 590 mμ using SPADNS reference solution as a reference. Prepare a plot of absorbance *versus* fluoride concentration using the absorbances of the sodium fluoride solutions. Determine the concentration of fluoride in the stannous fluoride solution using this plot. The amount of stannous fluoride is not less than 92.0% nor more than 108.0%.

Reagents.—*Solution A.*—Dissolve 3.16 Gm. SPADNS [4,5-dihydroxy-3(*p*-sulfophenylazo)-2,7-naphthalenedisulfonic acid, trisodium salt, Eastman No. 7309] in 550 ml. of water.

Solution B.—Transfer 0.133 Gm. of zirconium oxychloride octahydrate, $ZrOCl_2.8H_2O$, to a 500-ml. volumetric flask and dissolve in 50 ml. of water. Add 350 ml. of hydrochloric acid, fill to the mark with water, and mix.

SPADNS Reference Solution.—Dilute 50 ml. of *Solution A* with 500 ml. of water and 35 ml. of hydrochloric acid. The SPADNS reference solution is stable.

Zirconium-SPADNS Reagent.—Mix equal volumes of *Solutions A* and *B*. The resulting solution stored in an amber bottle is stable.

DOSAGE FORMS

Capsules

Identity and Purity Tests.—Capsules of stannous fluoride respond to the identity tests in the monograph for stannous fluoride.

Assays—*Stannous Ion.*—The stannous ion as determined by the procedure in the monograph for stannous fluoride is not less than 90.0% nor more than 110.0% of the labeled amount.

Fluoride Ion.—The fluoride ion as determined by the procedure in the monograph for stannous fluoride is not less than 90.0% nor more than 110.0% of the labeled amount.

Solutions

Solutions of stannous fluoride respond to the identity tests and assay methods described in the monograph for stannous fluoride. Solutions of stannous fluoride should be prepared from oxygen-free purified water and used immediately.

DISCUSSION

Nebergall and co-workers prepared stannous fluoride in 86% yield by reacting stannous oxide

moistened with oxygen-free water with a 10% molar excess of 48% hydrofluoric acid (2). This is

$$SnO + 2HF \rightarrow SnF_2 + H_2O \quad \text{(Eq. 1)}$$

an exothermic reaction from which the stannous fluoride crystallizes as the reaction mixture cools. Stannous fluoride is also prepared by reacting powdered tin with anhydrous hydrogen fluoride (3, 4).

From studies of stannous fluoride as an ingredient in solid fuels containing uranium or plutonium fluoride, Thamer and Meadows (5) found that dry stannous fluoride was unusually stable under essentially anhydrous conditions or those of low humidity. Molten stannous fluoride at 650° in an oxygen-free atmosphere in a mechanically-shaken, platinum-lined bomb did not undergo decomposition after 3 months. In the reverse reaction, tin and stannic fluoride in a similar bomb at 700° for 2 hours were substantially converted to stannous fluoride. In this work, the melting and the boiling points of stannous fluoride were reported as 213 ± 1° and 850 ± 5°, respectively. During the boiling point determination in a copper still, about 3% of the distillate was oxidized to the oxyfluoride, $SnOF_2$.

Stannous fluoride and the other stannous halide salts exhibit many similar physical and chemical properties (6). Stannous fluoride solutions probably contain Sn^{++}, SnF^+, $SnF_2°$, SnF_3^-; the existence of $SnF_4^=$ is considered to be possible but not likely. If an excess of fluoride is present in the solution, the SnF_3^- entity is more prevalent. The stannous fluoride in aqueous solutions undergoes hydrolysis to form the sparingly soluble stannous hydroxide which gives the solution a cloudy appearance. In this hydrolysis reaction, a number of entities, as indicated by Eq. 2, can exist. The sparingly soluble stannous hydroxide, represented for simplicity as $Sn(OH)_2$, may lose water to form stannous oxide. In the presence of oxygen or oxidizing agents, the stannous ion undergoes oxidation to form the stannic ion.

$$Sn^{++} \xrightarrow{OH^-} SnOH^+ \xrightarrow{OH^-} [Sn(OH)_2] \xrightarrow{OH^-} [Sn(OH)_3]^- \quad \text{(Eq. 2)}$$

Stannous fluoride is freely soluble in water (7, 8). The solubility of stannous fluoride in solutions maintained at temperatures from 0 to 106° (the boiling point) is a straight line relationship (Fig. 1) (7). The specific gravity of a saturated solution of stannous fluoride at 25° is about 1.51; thus a saturated solution at 25° contains about 63% stannous fluoride, weight by volume (63 Gm./100 ml.).

Typical pH values of freshly prepared stannous fluoride solutions are given in Table I.

In general, the pH of a stannous fluoride solution gradually decreases on standing, and this decrease is accompanied by the formation of insoluble hydrolysis products.

Acidic fluoride solutions can etch the surface of the glass electrode, thereby reducing the electrode life. This reaction (Eq. 3) can be a problem in

$$SiO_2 + 4HF \rightarrow SiF_4 + 2H_2O \quad \text{(Eq. 3)}$$

solutions containing 1% or more of stannous fluoride.

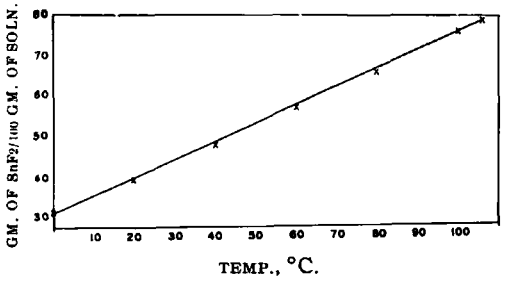

Fig. 1.—The solubility of stannous fluoride in water as a function of the temperature of the solution.

TABLE I.—pH OF FRESHLY PREPARED STANNOUS FLUORIDE SOLUTIONS

8%—2.3	2%—2.8
5%—2.5	1%—3.0
0.4%—3.2	

When the glass electrode is used to determine the pH of stannous fluoride solutions, the pH meter should be standardized with two buffer solutions. This procedure will reduce the possibility that a damaged glass electrode can be standardized at the pH of one buffer and still be inaccurate over a relatively small pH range. The sleeve-type reference electrode is preferred for measuring the pH of stannous fluoride solutions because the flow rate of electrolyte is much faster. There is a tendency for the small orifice containing the asbestos thread at the tip of the fiber-type reference electrode to become blocked. The pH of stannous fluoride solutions should be measured quickly to reduce the exposure of the electrodes to the solutions.

Stannous fluoride is somewhat more stable than stannous chloride and both are more stable in acidic solutions. The results of an early study with stannous chloride and fluoride are shown in Fig. 2. The solutions were freshly prepared and assayed immediately after the adjustment of the pH with sodium hydroxide. The assay procedure included centrifugation to remove the insoluble material,

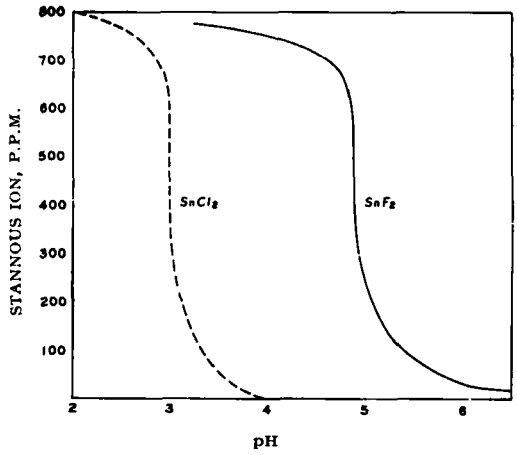

Fig. 2.—Stannous ion concentration of stannous chloride and fluoride (1000 p.p.m. Sn^{++}) solutions after adjustment of the pH of the solution with sodium hydroxide.

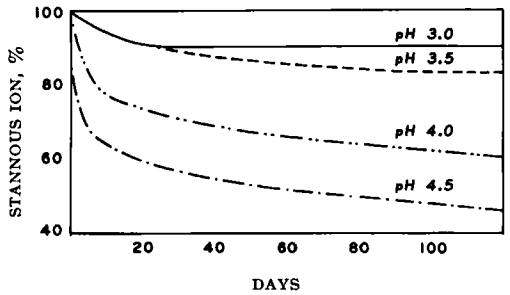

Fig. 3.—Effect of aging on stannous ion concentration in 0.4% aqueous solutions of stannous fluoride. The pH of the solutions was adjusted with sodium hydroxide.

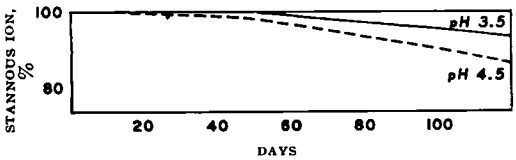

Fig. 4.—Effect of aging on stannous ion concentration of 0.4% stannous fluoride in an equal volume mixture of glycerin and water. The pH of the solutions was adjusted with sodium hydroxide.

then an iodine titration of the soluble stannous ion. In this early study, the soluble stannous ion content at the native pH of stannous chloride and fluoride was only about 85% of the theoretical value of 1000 p.p.m. This initial loss was probably because of a number of factors, including the purity of the particular samples of the stannous salts. As the pH's of the stannous chloride and fluoride solutions were raised from their native values around pH 3, the soluble stannous ion concentration dropped rapidly.

The effect of aging 0.4% aqueous solutions of stannous fluoride at different pH values is shown in Fig. 3. The pH of these stannous fluoride solutions was adjusted with sodium hydroxide, and the solutions stored in plastic bottles under an inert atmosphere. The plastic bottles then were stored in glass containers under an inert atmosphere. Here again, the greater stability of the soluble stannous ion in solutions at the native pH of stannous fluoride was demonstrated.

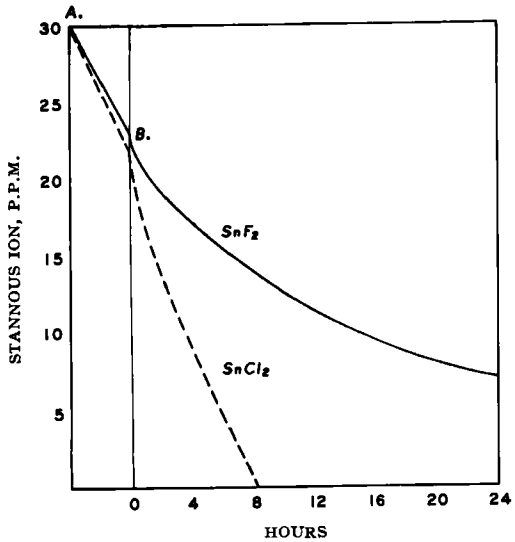

Fig. 5.—Stannous ion concentration of 0.0048% stannous chloride (30 p.p.m. Sn^{++}) and 0.004% stannous fluoride (30 p.p.m. Sn^{++}) at the time of dispensing into animal bottles and upon aging in these bottles. Key: A, initial concentration; B, concentration at time of dispensing.

The effect of adding glycerin or other highly water-soluble materials such as sugars, gums, etc., to stannous fluoride solutions is shown in Fig. 4. These stannous fluoride solutions were aged under the same conditions as those described in Fig. 3. When glycerin and these other materials are added, the activity of the free stannous ion is reduced; thus the rate of hydrolysis is reduced.

The stability data on the soluble stannous ion concentration in solutions of stannous fluoride in Figs. 3 and 4 were obtained from solutions prepared with degassed, distilled water stored in plastic containers under an inert atmosphere. With these conditions, the primary decomposition reaction is that of hydrolysis. The oxidation of the stannous ion is kept at a minimum. Stannous fluoride solutions stored without an inert atmosphere or in glass bottles decompose much more rapidly because three routes of decomposition are possible—hydrolysis, oxidation, and reaction with glass. In all such stability studies with stannous salts, it is important to centrifuge the solutions before assaying soluble stannous ion iodimetrically. The colloid-like, insoluble stannous hydroxide formed in a hydrolysis reaction is solubilized by the acidic iodimetric titration medium and thus is determined as soluble stannous ion.

As is true with most compounds, stannous fluoride is more stable in concentrated solutions. In animal experiments utilizing the drinking water as a route of administration, the exposure of very dilute solutions of stannous fluoride to the atmosphere can result in a significant loss in the soluble stannous ion. Francis (9) observed that the concentration of soluble stannous ion in mixtures containing an initial concentration of 30 p.p.m. decreased rapidly within a few hours (Fig. 5). In these experiments with rats, the solutions were prepared in the morning and transferred immediately to the usual liquid dispensing bottles of laboratory animal cages. As a result of the significant loss in soluble stannous ion concentration, it would be very easy to obtain misleading animal data. For this reason, Francis suggested that the stannous salts be administered topically rather than in the drinking water.

In all these stability studies, the concentration of soluble stannous ion was reported as a function of time, pH, or both. In these solutions, which contained essentially no foreign cations, the soluble fluoride remained relatively constant. Introduction of soluble calcium salts or calcium salts solubilized by the acidity of the solutions can significantly reduce the soluble fluoride ion concentration by the formation of calcium fluoride. The reduction in the soluble fluoride concentration reduces the overall complexing effect of the solution and therefore the stannous ions are more susceptible to hydrolysis.

Qualitative Tests.—There are many identity tests in standard chemistry texts for the stannous and fluoride ions of stannous fluoride. The two tests for stannous ion in the monograph are oxida-

tion-reduction reactions involving the oxidation of stannous to stannic ion and the reduction of silver and mercuric ions to the respective metals. By adding the stannous fluoride solution dropwise to mercuric chloride, it is possible to see the formation of the intermediate white mercurous chloride, which is then further reduced.

The reaction of fluoride with the cerium or lanthanum complex of alizarin complexan (3-aminomethylalizarin-N,N-diacetic acid) is one of the few, if not the only positive colorimetric response to fluoride ion. The alizarin complexan was prepared by Belcher and co-workers by a Mannich-type condensation of alizarin, iminoacetic acid, and formaldehyde (10). The alizarin complexan is now available from Burdick & Jackson Laboratories of Muskegon, Mich.

Polarography.—Polarography is one of the most important techniques for the detailed study of stannous fluoride alone and in combination with other agents. The hydrolysis of stannous fluoride is an example of a reaction which can be studied polarographically. The stannous ion in aqueous stannous fluoride solutions readily undergoes complex formation and partial or complete hydrolysis. Partial hydrolysis is a form of complexing in which the average number of hydroxyl groups bound per stannous ion is less than two. Polynuclear complexes involving Sn—OH—Sn bonds are frequent products of partial hydrolysis when no large excess of fluoride or other complexing anion is present.

Polarographically, freshly prepared stannous fluoride solutions (0.001 to 0.01 *M* stannous fluoride in 0.1 *M* potassium perchlorate) at or below their natural pH generally yield reversible cathodic waves at half-wave potentials of −0.45 to −0.50 volts (D.M.E. *versus* S.C.E.). Typical values of $i_d/Cm^{2/3}t^{1/6}$ are 3.7 to 4.4 at 25°. The presence of Sn-OH bonds and polynuclear complexes affects both the half-wave potential and the diffusion current. The polarographic behavior of any particular solution depends on the history of the stannous fluoride used—for example, its exposure to moist air, as well as the pH, ionic strength, and age of the solution. Aging of the solution or the presence of other complexing agents often leads to irreversible reduction waves.

Maxima sometimes observed in the reduction waves can usually be suppressed by addition of 0.001 to 0.01% gelatin. Complete suppression of the maxima sometimes lowers the overall diffusion current to a measurable degree.

With extremely rigorous control of manipulative variables, including careful exclusion of dissolved air from solutions, it is possible to use polarography for quantitative analysis of stannous ion in stannous fluoride solutions; however, the relatively simple iodimetric method is usually preferred. The main usefulness of polarography is in detecting and measuring changes in the nature of the soluble stannous species caused by complexing, including hydrolytic reactions.

The references (11–13) do not deal specifically with polarography of stannous fluoride but do contain extensive background information about the hydrolysis of stannous fluoride, fluoride complexes of stannous ions, and polarography of tin ions under various conditions.

Amalgam-Electrode Potentiometry.—Potentiometry with a tin amalgam electrode is a technique allied to polarography that has been useful in study of tin complexes (14). From a measurement of the equilibrium potential between stannous ions in solution and tin in the amalgam, the amounts of free and complexed stannous ions can be calculated. The situation is analogous to measurement of half-wave potentials in polarography. The potentiometric method has been used to measure the stabilities of polynuclear hydroxy complexes of tin in solutions of stannous perchlorate.

Purity Tests.—The purity test involving loss of weight on drying at 105° may be a measure of the hydrogen fluoride as well as the moisture content of the sample because hydrogen fluoride or hydrofluoric acid is used in the manufacture of stannous fluoride. The water-insoluble materials are usually the sparingly soluble hydrolysis products of stannous fluoride.

The metallic impurities in stannous fluoride can be determined spectrographically or colorimetrically (15). In the spectrographic procedure, the fluoride is usually removed by treatment with nitric acid in a platinum crucible. The usual limits placed on these materials in stannous fluoride are: iron–0.05%, nickel–0.05%, copper–0.01%, lead–0.005%, antimony–0.005%, and arsenic–0.0003%. The total of these materials should be less than 0.1%.

Assays.—The iodimetric determination of stannous ion is not a specific method; however, in most situations it is a convenient and generally applicable method (15). The titration depends upon the

$$Sn^{++} + I_2 \rightarrow Sn^{++++} + 2I^- \qquad \text{(Eq. 4)}$$

reaction of iodine with stannous ion to form iodide and stannic ions, respectively. The two commonly used iodine titrants are an iodine solution containing potassium iodide to solubilize and reduce the volatilization of the iodine and slightly alkaline iodate solution containing potassium iodate and iodide. The iodate-iodide reagent releases iodine

$$IO_3^- + 5I^- \xrightarrow{H^+} 3I_2 + 3H_2O \qquad \text{(Eq. 5)}$$

when added to the acidic titration medium (Eq. 5). The iodate titrant requires an acidic titration medium; the iodine titrant does not. Although the iodate reagent is generally considered to be somewhat more stable, both reagents should be stored in amber bottles and standardized at reasonable intervals.

Because the stannous ion is easily oxidized by the oxygen in air, it is important to carry out the titration in an inert atmosphere such as carbon dioxide or oxygen-free nitrogen. Specially purified nitrogen can be purchased as such or prepared by passing the nitrogen over heated copper turnings or through Fieser's hydrosulfite solution (16). A good quality deionized or distilled water should be used in all the assays. The water used in the preparation of the reagents should be made oxygen-free by boiling and cooling under an inert atmosphere or by degassing with purified nitrogen. Polarographic measurements suggest that the degassing procedure is somewhat more efficient in removing the oxygen.

Since there are a number of variables which can affect the iodimetric determination of stannous

fluoride, it is important to standardize the titrant using conditions and reagents as similar as possible to those used in the actual titrations. The most common method of standardizing the titrant is the use of reagent grade tin (15). Another standard which may sometimes be more convenient is sodium thiosulfate. The sodium thiosulfate reagent which has been standardized against arsenic trioxide or other suitable reference standard is especially useful in standardizing very dilute titrants.

Starch is the most common indicator for iodimetric titrations. The presence of detergents in the titration medium may interfere with the colorimetric end point by absorbing the starch. With these mixtures and others containing color interfering agents, the iodimetric titration can be followed potentiometrically with a potentiometer and a set of platinum and glass electrodes or a platinum combination electrode. This particular titration is quite suitable to a number of automatic and semiautomatic titration setups. Using the potentiometric or colorimetric starch end point, it is possible to titrate with iodine titrants with a normality as low as 0.005.

Since the iodimetric procedure is basically an oxidation-reduction procedure, any agent which can act as an oxidizing or reducing agent can interfere with the titration. A more specific method for the determination of tin is reaction with dithiol to form the red tin-dithiol complex which absorbs at about 530 mμ (15, 17).

The ion exchange method for the fluoride in stannous fluoride is a nonspecific assay which is useful in routine manufacturing control. In this method, the tin ion is reacted with the acid form of strongly acidic cation exchange resin such as Dowex 50W-X8 to release hydrogen ions. The hydrofluoric acid formed by the reaction is then titrated with standard base to the phenolphthalein end point. Because of the hydrofluoric acid formed in this reaction, plastic laboratory equipment should be used for this assay. Other halide salts and many other salts can interfere with the assay by participating in the exchange reaction with the resin.

A recent review describes the many methods which are available for the determination of fluoride (18). One of the major problems in the determination of fluoride is the separation of the fluoride from other interfering ions, such as calcium, phosphate, and sulfate, which are frequently present with fluoride in biological and other materials. Although ion exchange, paper chromatography, and other methods have been used for the separation of fluoride from interfering ions, the three most important procedures have been perchloric acid distillation, pyrolytic decomposition, and perchloric acid diffusion. The carefully controlled perchloric acid distillation of the fluoro-silicon compound usually described as fluosilicic acid (H_2SiF_6) has been one of the most widely used procedures (19). This procedure has been modified for micro work by utilizing nitrogen instead of steam as a carrier gas for the distillation, thereby greatly reducing the volume of distillate required for complete recovery of the fluoride (20). Separation by pyrolysis depends upon the hydrolytic decomposition of halides at about 1000° in the presence of superheated steam (21, 22). The most recent method of separation is the diffusion of hydrogen fluoride gas formed by the reaction of the

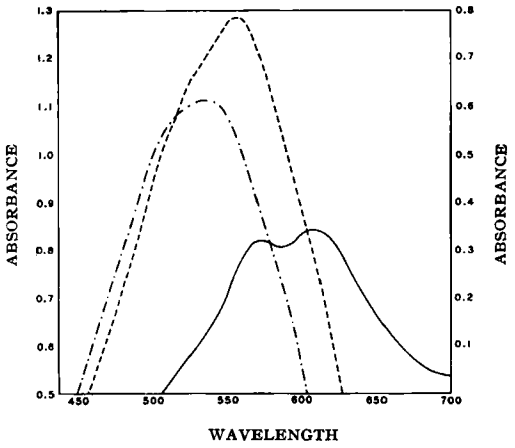

Fig. 6.—Spectra of lanthanum-alizarin complexan (reagent) and its fluoride adduct. Key: ——, reagent *vs.* water (abs. 0.5 to 1.5); - - - - -, reagent-fluoride *vs.* water (abs. 0.5 to 1.5): and ——, reagent-fluoride *vs.* reagent (abs. 0 to 1.0).

fluoride in the sample with perchloric acid (23). From the standpoint of equipment required and overall simplicity, the diffusion method, which utilizes plastic micro Conway dishes, is the preferable method of separating the fluoride. After the fluoride has been separated from the interfering ions, a wide variety of methods can be utilized for the determination.

The reaction of fluoride with lanthanum or thorium chloranilates has been utilized for fluoride determinations (24, 25). The insoluble chloranilate salts react with fluoride to form the soluble chloranilic acid and the corresponding insoluble fluoride salt. The concentration of the solubilized chloranilic acid is then determined by the absorbance at two different wavelengths depending upon the fluoride concentration, for example, 2 to 100 p.p.m., 530 mμ, 0.5 to 3 p.p.m., 330 mμ. While the chloranilate methods are not sensitive to most common anions, except for phosphate, the methods utilize a two-phase system with its consequent disadvantages.

The reaction of cerium or lanthanum alizarin complexan with fluoride is one of the few positive color reactions for fluoride. Unlike most bleaching methods involving a decrease in absorption as the fluoride ion concentration increases, the reaction of the cerium or lanthanum complex with fluoride results in an increase in absorption and a shift in wavelength of the maximum. The cerium complex of alizarin complexan (3-aminomethylalizarin-N,N-diacetic acid) is red with an absorption maximum in water at about 495 mμ (26). The reagent reacts with fluoride to form a blue-purple color with a maximum at about 567 mμ. The wavelength for the colorimetric determination is at about 610 mμ. At this wavelength, the maximum difference between the absorption spectra of the fluoride-containing complex and the cerium chelate occurs. The spectra of the corresponding lanthanum compounds are illustrated in Fig. 6. The wavelength of the maximum for this method and for any colorimetric method should be determined for each instrument and with each lot of dye. The stability of this

blue fluoride complex has been increased by using acetonitrile-water (1:5) mixture as a solvent for the color reaction (27).

The colorimetric methods which depend on the bleaching of the color of metal chromophoric combinations due to the stronger complexing of the metal ion by fluoride are older and generally more widely used fluoride methods. The zirconium-alizarin red S mixture utilizes color measurement at 520–525 mμ (28). The alizarin red S reaction product with fluoride has also been titrated with thorium nitrate, which depends upon the formation of the insoluble thorium fluoride (28). Two other zirconium lakes which have a low sensitivity to foreign ions and a high sensitivity to fluoride are the zirconium-eriochrome cyanine R (29) and zirconium-SPADNS [4,5-dihydroxy-3-(*p*-sulfophenylazo)-2,7-naphthalenedisulfonic acid, trisodium salt] (30). The SPADNS reagent, which is stable indefinitely, has increased sensitivity to fluoride when the molar ratio of zirconium to SPADNS is 1 to 12 (23).

Stannous ion does not interfere with the zirconium-SPADNS color reaction. Thus, it is possible to determine the soluble fluoride in a particular sample of stannous fluoride by simply dissolving the sample in water and diluting to a fluoride concentration ordinarily used in this procedure, 1–4 mcg./ml. When there is any doubt about the composition or contaminants of the sample, it is much safer to separate the fluoride from the rest of the sample before the determination.

Commercial detergents used for cleaning laboratory ware usually contain phosphate or sulfate ions, which interfere with most fluoride methods. Thus, it is important that the rinsing subsequent to washing the laboratory ware be adequate to remove all traces of these interfering ions.

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Some Possible Errors in the Plotting and Interpretation of Semilogarithmic Plots of Blood Level and Urinary Excretion Data

By JOHN G. WAGNER

Semilogarithmic plots of amount (or per cent) not excreted against time will be curved either if (a) the wrong asymptote is used, or (b) absorption is still proceeding during the interval observations are made. Factor (a) causes "convex decreasing" type of curvature, whereas factor (b) causes "concave decreasing" type of curvature. If both factors are operative, it is possible to obtain an apparently linear semilogarithmic plot; however, the rate constant estimated from the linear segment is meaningless. Models and actual data from the literature are used to illustrate these points. Using a simple model, it is shown that rate constants estimated from terminal segments of semilogarithmic plots of amount not excreted or of blood levels may be appreciably lower than the true values. The implications of these factors in making in vitro–in vivo correlations are discussed.

VAN LIEW (1) indicated that concavity[1] of a semilogarithmic curve may result from operation of one of four types of systems. Application of the "backward projection" technique to "convex decreasing" semilogarithmic plots of blood level and urinary excretion data can lead to linear components from which rate constants between assumed independent compartments may be estimated. Van Liew showed that another interpretation may be the operation of a continuum of exponential processes.

Assuming the independent compartment model applies to blood level and urinary excretion data, this report will discuss (*a*) other causes of curvature of semilogarithmic plots, (*b*) possible errors in plotting and interpreting such data, and (*c*) the implications of these factors in making *in vitro–in vivo* correlations.

Several types of semilogarithmic plots are commonly utilized. In these, time is plotted as the abscissa and the ordinates plotted on the logarithmic scale are (**A**) urinary excretion rate (amount/unit time or per cent of dose/unit time), (**B**) amount (or per cent) not excreted, and (**C**) blood (serum or plasma) concentration. Data plotted as the ordinate of Cartesian coordinate paper frequently are (**D**) cumulative amount or per cent of the dose excreted, (**E**) urinary excretion rate, and (**F**) blood (serum or plasma) concentration; again, time is plotted as the abscissa in each of these. On the semilogarithmic plots, curvature of the type discussed by Van Liew (1) is termed "convex decreasing" (analogous to Curve A in Fig. 3) to distinguish it from another type of curvature termed "concave decreasing" (analogous to segments AB in Figs. 1 and 2).

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[1] The terminology used in this report would describe the type of curvature Van Liew discussed as "convex decreasing." See Fig. 12 for the descriptions.

EXPERIMENTAL

Consider Model I:

$$X \xrightarrow[(0.180\,\text{hr.}^{-1})]{k_1} Y \xrightarrow[(0.090\,\text{hr.}^{-1})]{k_2} Z$$

Let us assume that (*a*) the rate constants, k_1 and k_2, are first-order rate constants and have the values shown; (*b*) 100% of the "drug" is initially in compartment X at time, $t = 0$; (*c*) the volumes of compartments X, Y, and Z are equal. This is an idealized model for first-order absorption and first-order elimination if X is considered to be the reservoir of "drug" at the absorption sites, Y is drug in the volume of distribution, and Z is drug lost by various processes from the volume of distribution. Figure 1 is a semilogarithmic plot of per cent of "drug" in Y against time based on the above model; it is analogous to type (**C**) plot, above. The plot has pronounced "concave increasing" curvature during the interval when most of the drug is leaving X. Although the curve is actually continuous, only some of the values have been calculated and the points joined; this is the case in other examples shown too. At point A only 13.8% and at point B only 2.28% of the "drug" is left in X. It would be easy to interpret segment AB as a straight line, especially if the assay error for the drug were relatively large as is often true with blood assays. The segment BC appears linear even with the idealized values obtained from the model. Estimation of k_2 from the segment BC gave a value of 0.080 hours^{-1} which is 11% lower than the actual value of 0.090 hours^{-1}.

Using Model I, a type (**B**) plot is shown as Curve I in Fig. 2. The per cent of "drug" not in compartment Z would be analogous to per cent of drug not excreted if all the drug initially in X were excreted unchanged in the urine, Z. Note that Curve I is "concave decreasing" from A to B. For times beyond B the plot appears linear. Estimation of k_2 from the segment BC gave a value of 0.0866 hours^{-1} which is 3.8% lower than the actual value of 0.0900 hours^{-1}. This example indicates that estimation of k_2 from "urinary data" (sampling in Z) is more accurate than estimation of k_2 from "blood level data" (sampling in Y) in the same time interval. In actual practice the higher concentrations of drug in urine than in blood and the greater accuracy and precision of urine assays compared with blood assays also contribute to greater accuracy from urine data than blood data.

When constructing type (**B**) plots, values of ($Ae°$-Ae) must be calculated where $Ae°$ is the amount of drug excreted in the urine at "infinite time" (about ten half-lives is usually an adequate collection time). The ($Ae°$-Ae) values or the percentages $Ae°$-$Ae/Ae° \times 100$, are plotted on the logarithmic axis. $Ae°$ is called the asymptote of the first-order plot. Some authors incorrectly use the administered dose for the value of $Ae°$ when they do not show experimentally that the dose is excreted completely in the urine. An example of use of such an incorrect asymptote is shown as Curve II in Fig. 2. Curve II was constructed by assuming that the "dose" initially in X of Model I was 125 mg. but that only 100 mg. of the intact "drug" appeared at infinite time in Z; however, rather than using the correct value for $Ae°$, namely 100 mg. as in Curve I, the incorrect value of 125 mg. (the "dose") was utilized to calculate the percentages. Curve II is "concave decreasing" from D to E but becomes apparently linear from E to F; from F to G the curve is "convex decreasing." One could interpret that in such a plot the segment EF represents first-

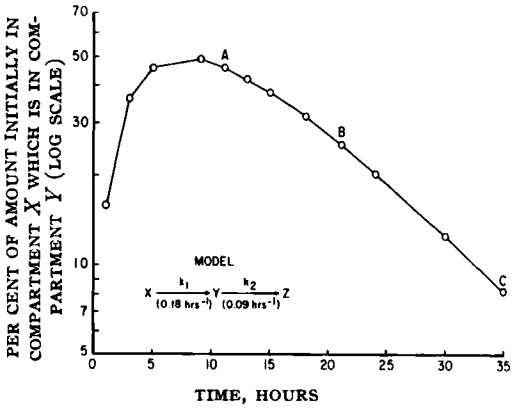

Fig. 1.—Semilogarithmic plot of per cent of "drug" in Y against time based on Model I.

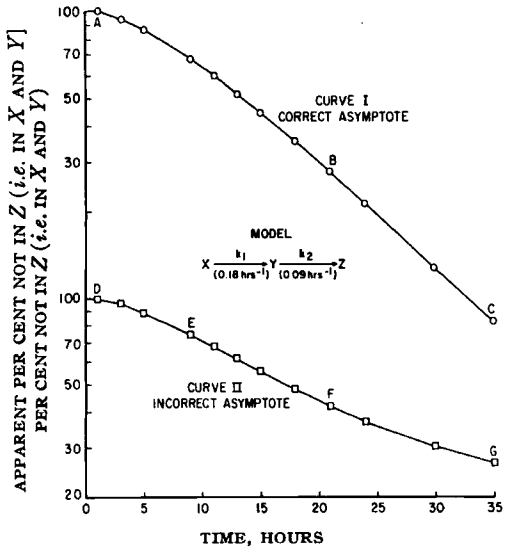

Fig. 2.—Semilogarithmic plot of per cent (-o-) and apparent per cent (-□-) not in z against time based on model I.

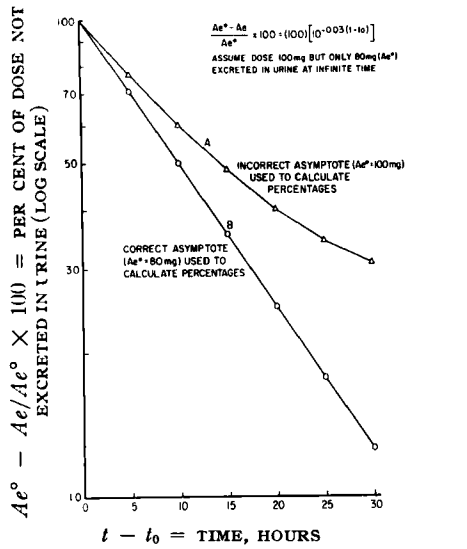

Fig. 3.—Semilogarithmic plot of Eq. 1; k = 0.03 for both Curves A and B.

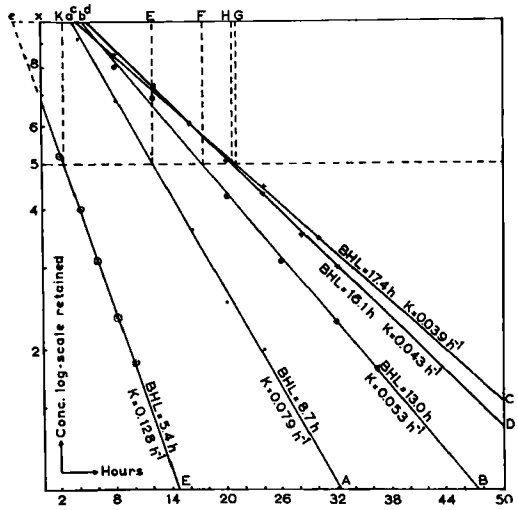

Fig. 4.—Reproduction of Fig. 31 of Simoons (2).

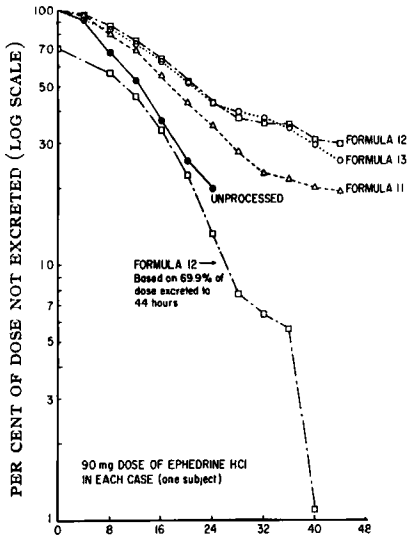

Fig. 5.—Tabular data (2) of Fig. 4 replotted.

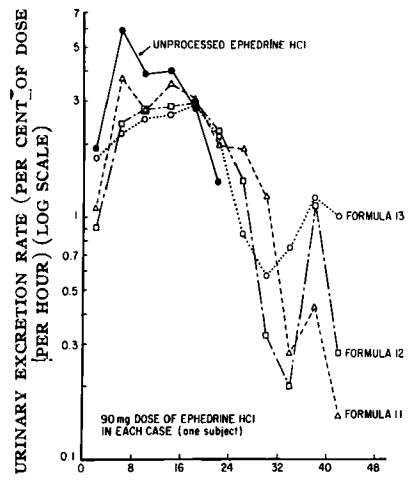

Fig. 6.—Type (A) plots derived from data in Figs. 4 and 5.

order loss of drug from the body or appearance in the urine, particularly if little or no data were collected beyond point F. The rate constant estimated from segment EF is 0.0481 hours^{-1} which is approximately one-quarter and one-half of the actual values of k_1 and k_2, respectively. The rate constant derived from segment EF is purely "artificial" and bears no real relationship to either of the rate constants k_1 and k_2. In this example the effect of "absorption still proceeding" (*i.e.*, transfer of significant amounts of "drug" from X to Y), which causes "concave decreasing" curvature,[2] and the effect of using the wrong asymptote, which causes "convex decreasing" curvature, cancel each other. The net effect is an apparently linear segment which is completely artificial.

Consider Eq. 1

$$\frac{Ae^\circ - Ae}{Ae^\circ} \times 100 = (100)\{10^{-k(t-t_0)}\} \quad \text{(Eq. 1)}$$

This equation, according to theory, should fit urinary excretion data in two situations: (*a*) where t_0 is the end of the distribution-tissue equilibration phase following intravenous administration of the drug, or (*b*) where t_0 is the end of the absorption and tissue equilibration phase following oral administration and where $t > t_0$ in both cases. Figure 3 is a semilogarithmic plot of Eq. 1 with k equal to 0.03 for both Curves A and B. Assume the dose is 100 mg. but that only 80 mg. of drug appears in the urine in infinite time. Curve B was constructed using the correct asymptote, namely $Ae^\circ = 80$; the line is straight and has a slope of -0.03. Curve A was constructed using Ae° = dose = 100. Note that Curve A is "convex decreasing." Rate constants estimated from the points between 0 and 15 hours or between 15 and 30 hours would be greatly in error.

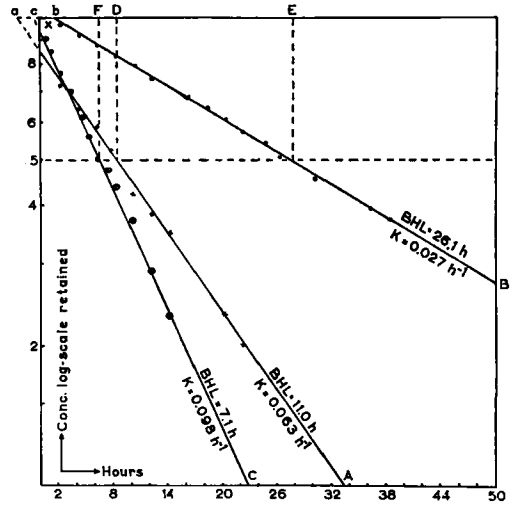

Fig. 7.—Reproduction of Fig. 32 of Simoons (2).

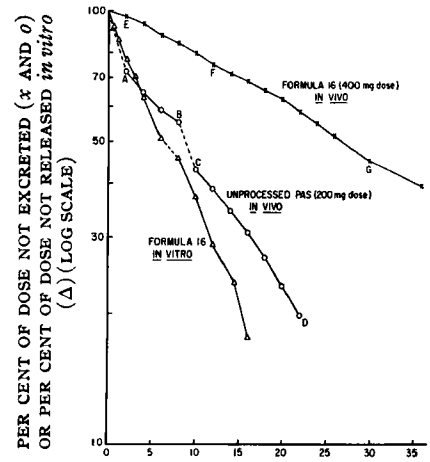

Fig. 8.—Tabular data of Fig. 7. replotted.

[2] "Concave decreasing" curvature may also be observed if the drug elimination process is zero order (rather than first order) and the blood concentration is plotted against time on semilogarithmic paper. In such a case, replotting the data on Cartesian coordinate paper should yield a linear plot of blood level against time.

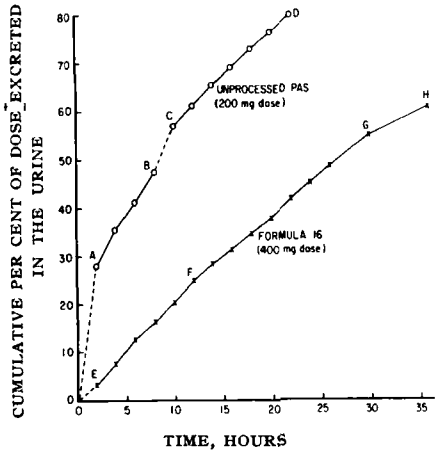

Fig. 9.—Type (**D**) plots derived from data in Figs. 7 and 8.

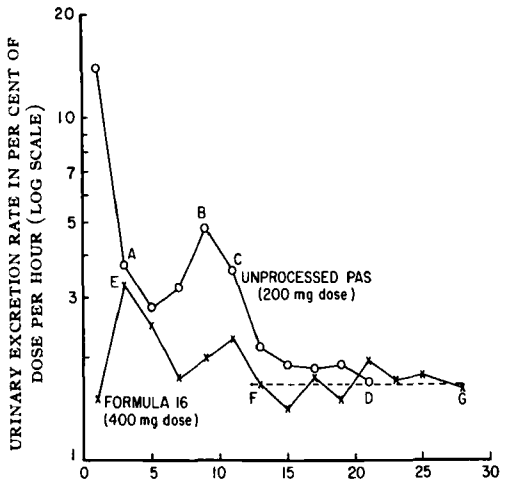

Fig. 10.—Type (**A**) plots for data shown in Figs. 7, 8, and 9.

Practical examples of the operation of some of the factors discussed above are found in the plots and data of Simoons (2). At different times a human subject was administered 90-mg. doses of ephedrine hydrochloride as unprocessed drug and in the form of three different sustained-release formulations. Urine was collected at regular intervals and analyzed for drug content. Formula 13 was tested *in vitro* using a new apparatus.[3] Figure 4 is a reproduction of Fig. 31 of Simoons (2). The tabular data (2) on which Fig. 4 was based were replotted and are shown in Fig. 5. In Fig. 5 the data points were merely joined, whereas in Fig. 4 straight lines were drawn through the points and extrapolated; data points have been left off the extrapolated regions of Fig. 4. The situation in Fig. 5 is analogous to Curve II in Fig. 2. No meaningful rate constant(s) can be calculated from any of the plots shown in Fig. 5. Also, since Ae° was not determined in any of the experiments (since urine was not collected long enough), a type (**D**) plot (such as Figs. 4 and 5) should not be made at all for data. Type (**A**) plots, derived from the same data, are shown in Fig. 6. The excretion rates are plotted against the midpoints of the urinary collection intervals over which the rates were calculated. Simoons (2) and Cummings and Martin (3) plotted the rates against the times at the end of the collection intervals; this distorts the plots and leads to erroneous conclusions. The peculiar curvature at the tail ends of the plots shown in Fig. 6 are not readily explained but may be because of continued irregular absorption and/or a recycling phenomenon. Although Fig. 6 is a "valid" plot for these data, it is obvious that no meaningful rate constants can be estimated.

Another example is taken from Simoons (2). At different times a human subject was administered 200 mg. of unprocessed *p*-aminosalicylic acid (PAS) and 400 mg. of PAS in the form of ten sustained-release tablets (formula 16). The latter were also tested *in vitro* using the new apparatus.[3] Figures 7–11 are derived from the same data given by Simoons (2). Figure 7 is a reproduction of Fig. 32 of Simoons (2). The tabular data (2), on which Fig. 7 were based, were replotted and are shown in Fig. 8. In Fig. 8 the data points were merely joined, whereas in Fig. 7 straight lines were drawn through the points. Inspection of the plot of the *in vitro* data in Fig. 8 indicates there are two essentially linear segments, one between 0.5 and 6 hours, the other between 8 and 16 hours, with a discontinuity between them from 6 to 8 hours; this is a different interpretation than the single linear plot shown in Fig. 7. Inspection of the plots of the *in vivo* data in Fig. 8 indicates curvature in the segments EF, FG, AB, and CD, with a discontinuity from B to C. No meaningful rate constants can be estimated from these *in vivo* data as was done by Simoons in Fig. 7. Again, since Ae° was not determined in either of the *in vivo* experiments, type **B** plots (such as Figs. 7 and 8) should not be made at all for these data. The type **D** plots for these data, shown in Fig. 9, support this statement; at times D and H, when urine collection ceased, excretion of drug in the urine was incomplete, but one could not predict that the entire dose (100%) would be excreted. Note that the segmentation of the plots shown in Fig. 8 is also apparent in Fig. 9. A type **A** plot is more "sensitive" than either types **B** or **D**. The type **A** plots for the same data are shown in Fig. 10. These plots show double peaks indicative of a recycling phenomenon. The plot for the unprocessed PAS did not become linear during the observation period; hence, no rate constant for loss of drug from the body can be calculated. Formula 16 gave an essentially constant excretion rate averaging 1.67% of dose/hour or 6.68 mg. of PAS/-hour in the 13 to 28-hour interval. This is a considerably different interpretation than the first-order rate constant of 0.027 hours^{-1} shown on Fig. 7. The variability about the mean rate in the 13- to 28-hour interval is exaggerated due to the logarithmic scale in Fig. 10; this variability is not nearly so evident in the type (**E**) plot of the same data shown in Fig. 11. Figure 11 has a considerably different appearance than Fig. 25 of Simoons (2), yet both were derived from the same tabular data.

[3] The apparatus is described in Reference 2. A commercial model, the Erweka Tester Type AT-3, is distributed by the Chemical and Pharmaceutical Industry Co., Inc., New York, N. Y.

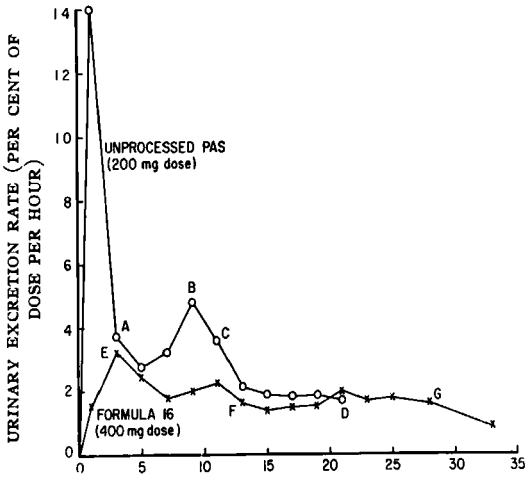

Fig. 11.—Type (E) plots derived from tabular data of Fig. 25 of Simoons (2).

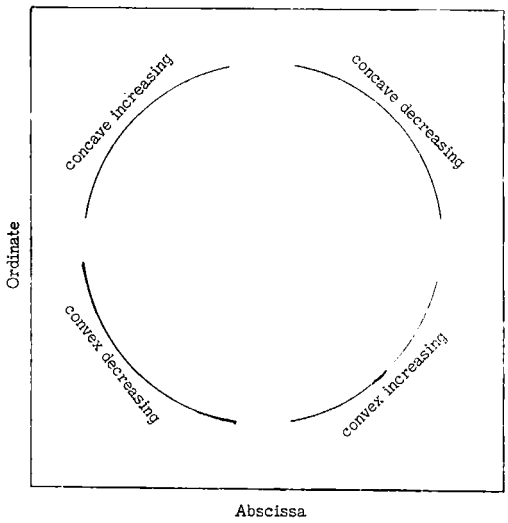

Fig. 12.—Description of concavity of semilogarithmic curve. The definitions assigned to the curve are those of James and James (6).

DISCUSSION

Given a set of urinary excretion data for interpretation, one should first make two types of plots: (*a*) a plot of cumulative amount excreted to time *t* against time *t*, and (*b*) a semilogarithmic plot of excretion rate (amount/unit time) against the midpoints of the urinary collection intervals.[4] If the first plot indicates an asymptote was reached, then (*c*) a semilogarithmic plot of amount not excreted against time should also be made. Theory (4) predicts that plots (*b*) and (*c*) should have terminal linear segments and that the rate constant derived from these linear segments should be same and equal to the rate constant for loss of drug from the volume of distribution. Care should be used to insure that the segments used do not show curvature and that "artifacts" are not produced by using the wrong asymptote in the third type of plot. Comparison of the three types of plots prepared from the same set of data allows more accurate interpretation and permits comparison of different segments as has been done in the figures in this report. Even in the absence of evidence of curvature in the terminal segments of the semilogarithmic plots one should be aware that the most likely situation is that the estimated rate constant will be less (the half-life greater) than the true value. This is also true of semilogarithmic plots of blood level against time.

The rate constant for clearance (or biological half-life) of a drug in the body is independent of dosage form effects if the data are properly collected and interpreted. Apparent deviation from this independence is purely an artifact due to inadequate data or misinterpretation of data. It is the author's experience that the rate constant for clearance (or biological half-life) of a drug in the body is almost always independent of the route of administration in a given species if the data are properly collected and interpreted. However, there are physiologic reasons for differences with some drugs.

The magnitude and duration of blood levels and urinary excretion of a drug can be markedly altered by dosage form effects, as many investigators have shown (5). An interesting and necessary area of research is to try to correlate *in vivo* and *in vitro* results obtained with different dosage forms. This research is "interesting" since correlation of *in vivo* results with physical constants such as rate of dissolution, solubility, and partition coefficient may someday lead to the ability to predict behavior of new drugs and old drugs in new dosage forms or at minimum reduce the number of biological experiments necessary and aid in experimental designs. Such research may someday be necessary for adequate control of pharmaceutical products, but we do not have enough knowledge today to apply such criteria adequately and rigorously.

It is obvious from the experimental part of this report that the apparent correlations of *in vitro* and *in vivo* results of Simoons (2) were only apparent and not real. In fact, it is completely spurious to correlate rate constants derived from *in vitro* dissolution tests with rate constants for clearance (or biological half-lives) of the drugs derived from blood level and urinary excretion data. If dissolution of the drug from its dosage form *in vivo* partly, or completely, rate limits its absorption, then the "correlation" should involve the use of blood level or urinary excretion data in the time interval shortly after administration. However, it is necessary to know the rate constant for clearance to perform the calculations (4, 5). Quantitative correlations are needed in this research area. The author trusts this report illuminates some of the pitfalls.

[4] If the cumulative curve is fitted by a polynomial and the derivatives estimated at the data points, then the excretion rate (the derivative) should be plotted against the time at the end of the collection intervals.

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Synthesis and Activity of 1-Alkyl-2-aryl-2,3-dihydro-4(1H)-quinazolinones

By HARVEY GURIEN† and BERNARD B. BROWN

Only one 1-alkyl-2-aryl-2,3-dihydro-4(1H)-quinazolinone has previously been reported, 1-methyl-2-phenyl-2,3-dihydro-4(1H)-quinazolinone (1). We have prepared a series of such novel quinazolinones and tested them for analgesic and sedative activity. These substances were obtained by reaction of the appropriate *o*-alkylaminobenzamide with an aromatic aldehyde in ethanol containing a small amount of hydrochloric acid according to the scheme

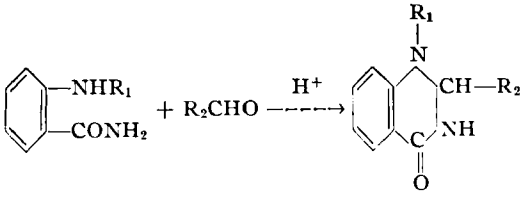


Table I summarizes the physical properties of these substances.

EXPERIMENTAL

1-Alkyl-2-aryl-2,3-dihydro-4(1H)-quinazolinones.—As a general procedure, 0.038 mole of the *o*-alkylaminobenzamide was mixed with 0.050 mole of aldehyde in 30 ml. of ethanol containing one drop of concentrated hydrochloric acid. The mixture was then refluxed for 36 hours. Removal of the solvent was carried out at reduced pressure and the residual solid was recrystallized from toluene. Substituted *o*-alkylaminobenzamides were obtained by reaction of the corresponding isatic anhydrides with ammonia.[1]

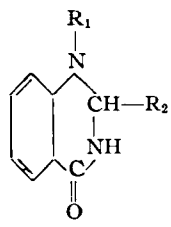
PHARMACOLOGICAL DATA[2]

None of the compounds tested showed any significant analgesic, sedative, or muscle relaxant activity in mice at levels up to 100 mg./Kg. when dosed intravenously using propylene glycol–water suspensions. Prostration and decreased activity were observed in the test animals when compounds IV, V, VI, and VII were administered at levels of 100–300 mg./Kg.

REFERENCES

(1) Böhme, H., and Böeing, H., *Arch. Pharm.*, **283**, 1011 (1960); through *Chem. Abstr.*, **55**, 7420d(1961).

TABLE I.—2-SUBSTITUTED-1-ALKYL-2,3-DIHYDRO-4(1H)-QUINAZOLINONES



Compd. No.	R_1	R_2	Yield, %	M.p., (corrected)	Formula	Analyses[a] Calcd. N	Calcd. S	Found N	Found S
I	Methyl	2-Furyl	73.5	184.2 to 185.6	$C_{13}H_{12}N_2O_2$	12.29	...	12.18	...
II	Methyl	3-Indolyl	56.4	217–220	$C_{17}H_{15}N_3O$	15.15	...	15.03	...
III	Methyl	2-Thienyl	90.0	178.8 to 181.2	$C_{13}H_{12}N_2OS$	11.46	13.11	11.00	12.82
IV	Methyl	Phenyl	62.9	206 to 207.5 (uncorrected)	$C_{15}H_{14}N_2O$	11.76	...	11.79	...
V	*n*-Propyl	2-Furyl	53.2	150.5 to 152.0	$C_{15}H_{16}N_2O_2$	10.91	...	10.30	...
VI	*n*-Propyl	Phenyl	49.0	120–121	$C_{17}H_{18}N_2O$	10.52	...	10.82	...
VII	*n*-Propyl	2-Thienyl	48.9	146–148	$C_{15}H_{16}N_2OS$	10.28	11.74	10.03	11.70
VIII	Ethyl	Phenyl	72.5	141 to 143.5	$C_{16}H_{16}N_2O$	11.10	...	10.95	...
IX	Isopropyl	Phenyl	52.2	122–125	$C_{17}H_{18}N_2O$	10.50	...	10.52	...
X	*n*-Butyl	Phenyl	85.8	111 to 112.5	$C_{18}H_{20}N_2O$	10.00	...	9.94	...
XI	*n*-Pentyl	Phenyl	85.5	123.5 to 126	$C_{19}H_{22}N_2O$	9.51	...	10.12	...

[a] Analyses were carried out by Spang Microanalytical Laboratories, Ann Arbor, Mich.

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Accepted for publication February 19, 1963.
† Present address: Hoffmann-La Roche, Inc., Nutley, N. J.

[1] *o*-Alkylaminobenzamides were prepared by C. Baumann in this laboratory (unpublished work).

[2] Tests carried out by Woodard Research Corp., Herndon, Va.

Adsorption of Certified Dyes by Starch

By GEORGE ZOGRAFI† and ALBERT M. MATTOCKS

The adsorption of five anionic certified dyes, FD&C Red No. 3, FD&C Blue No. 2, FD&C Green No. 1, FD&C Yellow No. 5, and Ext. D&C Red No. 15, by rice starch, wheat starch, and three varieties of cornstarch, was measured. Adsorption was found to fit the Langmuir equation in all cases. The extent of adsorption by the various cornstarches is related to the ratio of amylopectin to amylose, an increase in adsorption occurring with an increase in the amylopectin content. No dye adsorption was observed when potato starch was used. This is believed to be because of the presence of phosphate ester groups not present in the other starches.

ONE OF THE most troublesome applications of dyes in pharmaceutical products is the coloring of compressed tablets. It is generally found that the wet mass is uniform in color, but that the large granules, after drying, have a high concentration of dye at the surface and a low concentration beneath the surface. Thus, dye migrates toward the outside during drying and compression of this mixture, causing spotted tablets. At the present time, although pigments have been reported to yield evenly colored tablets (1, 2), there is no general means for predicting the facility of migration or preventing the migration of water-soluble dyes during tablet manufacture.

It was felt that studies concerned with dye affinity for solids would be of value to those concerned with this problem since an improvement of dye-solid affinity should reduce migration. This report is concerned with the affinity of selected water-soluble anionic, certified dyes for various natural starches. Starch was chosen since it is widely used in tableting, is available from a variety of different natural sources, and is insoluble in water at moderate temperatures.

EXPERIMENTAL

General Procedure for Measurement of Adsorption.—Adsorption studies were conducted using Kimax, heavy duty, screw-capped centrifuge tubes (Kimble Glass Co.) which have a capacity of about 40 ml. Tin-lined screw caps (Arthur H. Thomas) were used since they were found not to adsorb dye. A rubber ring was placed around the neck of the tube to prevent leakage. To maintain constant temperature and thorough mixing, the tubes were placed on a rotating wheel in a water bath at 30 ± 0.1° and were continuously rotated at 32 r.p.m. Approximately 1 Gm. of starch, accurately weighed, and 20 ml. of dye solution were placed into the tubes. These were placed in the bath and removed after 48 hours, since this was previously determined to be the time required to ensure equilibrium. The tubes were centrifuged at 2500 r.p.m. for 5 minutes, and a 10-ml. aliquot of dye solution was removed. Controls containing only dye solution were treated in the same manner and more than one sample for each dye concentration was used.

Dyes Investigated.—Since there are a large number of water-soluble certified dyes, those chosen for study are representative of the chemical groups into which most of these dyes fall. Ext. D&C Red No. 15 (formerly FD&C Red No. 1) was chosen as an azo dye, FD&C Green No. 1 as a triphenylmethane dye, FD&C Yellow No. 5 as a pyrazalone dye, FD&C Blue No. 2 as an indigo dye, and FD&C Red No. 3 as a xanthine dye. All dyes were obtained from the Calco Chemical Division, American Cyanamid Co., and were used as received.

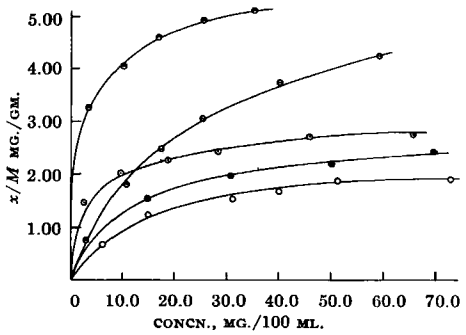

Fig. 1.—Plots of milligrams of dye adsorbed per gram of cornstarch, (x/M), *vs.* milligrams of dye per 100 ml. of solution at equilibrium for FD&C Red No. 3 (●), FD&C Blue No. 2 (⊖), Ext. D&C Red No. 15 (⊙), FD&C Yellow No. 5 (⊕), and FD&C Green No. 1 (○).

The concentration of dye in solution was measured spectrophotometrically using a Beckman model DU spectrophotometer. Absorbance measurements were made at 504 mμ for Ext. D&C Red No. 15, 527 mμ for FD&C Red No. 3, 426 mμ for FD&C Yellow No. 5, 620 mμ for FD&C Blue No. 2, and 428 mμ for FD&C Green No. 1. The final dilution of all dyes, except FD&C Red No. 3, was made into a phosphate-citrate buffer at a pH 5.00, while the final dilution of FD&C Red No. 3 was made into a borate buffer at pH 9.20. The pH values were measured using a Beckman model G pH meter.

Starches Investigated.—Starches utilized in this investigation included those from potato, rice, wheat, and three varieties of corn. The cornstarches included U.S.P. cornstarch containing about 72% amylopectin and 28% amylose, waxy maize starch commercially available with the brand name, Amioca,[1] and containing 100% amylopectin, and Amylon,[1] a cornstarch containing about 60% amylose and 40% amylopectin.

RESULTS

From the difference in dye concentration before and after contact with starch, the number of milligrams of dye adsorbed per gram of starch, x/M, was calculated for each concentration of dye used.

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† Present address: College of Pharmacy, Columbia University, New York, N. Y.

[1] Obtained from National Starch and Chemical Corp., New York, N. Y.

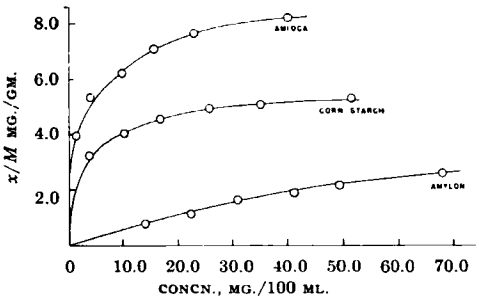

Fig. 2.—Plots of milligrams of FD&C Red No. 3 adsorbed per gram of starch, (x/M), *vs.* milligrams of dye per 100 ml. of solution at equilibrium for three varieties of cornstarch.

Adsorption of all dyes occurred on all starch samples except potato starch, which did not adsorb these dyes. Figure 1 shows a typical plot of x/M *versus* C, the concentration of dye in solution at equilibrium, for all five dyes on cornstarch. Figure 2 shows the marked differences in adsorption for a particular dye on the three cornstarches. The data obtained were found to fit the Langmuir adsorption equation

$$x/M = \frac{k_1 k_2 C}{1 + k_1 C} \qquad \text{(Eq. 1)}$$

where k_1 and k_2 are constants. This is shown in Fig. 3 by the fit of the data to the linear form of this equation

$$C/(x/M) = \frac{C}{k_2} + \frac{1}{k_1 k_2} \qquad \text{(Eq. 2)}$$

From plots of $C/(x/M)$ *versus* C the constants, k_1 and k_2, were evaluated as shown in Table I. The constant, k_2, expressed in milligrams adsorbed per unit mass of adsorbent, is the maximum value for x/M at a given temperature. This can be seen by applying the Langmuir equation at high concentrations, where the value of k_1C becomes much greater than 1, and

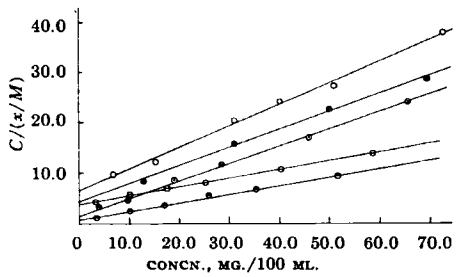

Fig. 3.—Langmuir plots for the adsorption of dyes on cornstarch; FD&C Red No. 3 (●), FD&C Blue No. 2 (⊖), Ext. D&C Red No. 15 (⊙), FD&C Yellow No. 5 (⊕), and FD&C Green No. 1 (○).

$$x/M = k_2 \qquad \text{(Eq. 3)}$$

The constant, k_1, expressed in 100 ml./mg.$^{-1}$ indicates the fraction of maximum adsorption per unit concentration. This is apparent at low concentrations where C may be considered to be much less than 1 and

$$\frac{x/M}{k_2} = k_1 C \qquad \text{(Eq. 4)}$$

DISCUSSION

A comparison of the constants, k_1 and k_2, indicates that there is little difference in adsorption of a particular dye on corn, rice, and wheat starches, although the particle sizes are quite different (3). Calculation of the maximum number of dye molecules adsorbed, utilizing k_2 values, indicates more adsorption than is accountable for on the basis of surface adsorption on the starch grains. This, plus the fact that 48 hours is required to reach equilibrium, suggests that penetration of the various dyes into the starch grain is occurring. As has been shown with cellulose (4), the adsorption probably occurs at the hydroxyl groups of the starch granule. Whether the anionic or the nonionic polar group of the dye is responsible for this interaction has not been definitely determined. Hydrogen bonding between nonionic polar groups of a dye

TABLE I.—LANGMUIR CONSTANTS[a] FOR ADSORPTION OF DYES ON VARIOUS STARCHES[b]

Starch	FD&C Blue No. 2	Ext. D&C Red No. 15	FD&C Yellow No. 5	FD&C Red No. 3	FD&C Green No. 1
Rice					
k_1[c]	0.118	0.146	0.100	0.226	0.0318
k_2[d]	3.55	3.18	2.71	6.64	1.03
Wheat					
k_1	0.155	0.158	0.123	0.223	0.0808
k_2	3.15	3.02	2.47	6.20	1.73
Corn					
k_1	0.0438	0.195	0.0916	0.284	0.0644
k_2	5.85	2.94	2.75	5.67	2.30
Amylon					
k_1	0.231	...	...	0.223	...
k_2	2.62	...	...	2.57	...
Amioca					
k_1	0.0241	0.231	0.195	0.337	0.0419
k_2	11.6	4.19	3.27	8.86	4.65

[a] On the basis of the dry weight of the starches. [b] Blank spaces indicate little or no adsorption. [c] Units of 100 ml./mg. [d] Units of mg./Gm.

and cellulose hydroxyl groups has been proposed (4).

A comparison of the adsorption by the three varieties of cornstarch indicates that Amioca adsorbed all dyes to the greatest extent, U.S.P. cornstarch next, and then Amylon. This trend is related to the amylopectin content. Recent studies concerned with anionic dye binding of soluble starches have shown the opposite results (5): the less interaction occurring, the higher the proportion of amylopectin. This is attributed to the greater rigidity of the branched portions of starch in solution compared to the more flexible linear amylose chains. One would not necessarily expect interactions involving the intact starch grain to be the same as that in solution. The results presented here confirm this.

The lack of anionic dye adsorption on potato starch is apparently because of the presence of phosphate esters (6) which impart a negative charge to the potato starch grain, but which are absent in the other starches. Schoch and Maywald (7) have shown that potato starch and carboxylated starches adsorb cationic dyes strongly but do not adsorb anionic dyes. Adsorption of anionic dyes on potato starch has been observed when neutral electrolytes are added (8). These would be expected to reduce the repulsive forces between the dye and starch by breaking down the electric double layer.

In view of the observed affinity of anionic certified dyes for starch it would appear that the addition of starch to a tablet granulation before the addition of the dye solution should aid in preventing color migration. A recent communication (9) has indicated that starch does enhance color distribution and prevent migration when added to tablet granulations.

In general, it is proposed that adsorption isotherm data can be used to study color migration problems. These isotherms indicate the amount of dye adsorbed as a function of that amount remaining in solution. Since it is the dye in solution which migrates upon drying, the objective should be to minimize this concentration while increasing the amount adsorbed. For systems described by the Langmuir equation the constants, k_1 and k_2 can be considered as a measure of the extent of adsorption for a given dye concentration and the maximum amount of adsorption, respectively. Therefore, by measuring adsorption as a function of such factors as temperature, solvent, electrolyte concentration, and the presence of tablet components, it should be possible to pick systems giving maximum dye adsorption with a minimum of dissolved dye, resulting in maximum color distribution.

Further studies are being conducted and will be reported in a future communication.

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(7) Schoch, T. J., and Maywald, E. C., *Anal. Chem.*, 28, 382(1956).
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Reduction of 1,2,3-Trimercaptopropane Content of Dimercaprol

By EDWARD G. RIPPIE† and ALBERT A. KONDRITZER

A multiple batch extraction procedure is described which can serve to reduce the trithiol content of dimercaprol to levels conforming to the U.S.P. XVI monograph.

Previous reports (1, 2) indicate the presence of the trithiol, 1,2,3-trimercaptopropane, in many laboratory, pilot plant, and commercial lots of dimercaprol (BAL). The toxicity of BAL is substantially increased by this impurity and the revised monograph on dimercaprol appearing in the first U.S.P. XVI supplement contains limits on the concentration in which it may be present. Many lots of BAL, presently available for drug use, contain far more than the established 1.5% limit of the trithiol. Since BAL is not currently manufactured in this country, a purification process is needed.

This report presents the results of a study of the removal of 1,2,3-trimercaptopropane from BAL by a liquid-liquid extraction procedure, using petroleum ether as the extracting solvent. This method may be used with a variety of solvents under various experimental conditions, *e.g.*, BAL saturated with water to increase the partitioning of the trithiol into the organic solvent phase to obtain basic information of value for the development of a commercial process for the purification of BAL containing excessive quantities of the trithiol impurity.

EXPERIMENTAL

Materials.—BAL purified by passage through a partition chromatographic column, followed by distillation at low pressure; 1,2,3-trimercaptopropane, hereinafter designated as TSH; petroleum ether, shaken with concentrated sulfuric acid over a period of several days and distilled between 35–50° were employed.

Procedure.—Known concentrations of TSH in BAL were equilibrated with petroleum ether. The volume of the BAL was chosen sufficiently large so that it was not significantly changed by the extraction. The petroleum ether phase was analyzed for sulfhydryl content, and the resultant data utilized in calculations of extraction efficiency.

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TABLE I.—RESULTS OF EXTRACTION OF TSH FROM BAL WITH PETROLEUM ETHER

1	2	3	4	5	6	7	8
	Before Extn.		Iodine Titer per ml. Petroleum Ether		TSH mg./ml. Petroleum		
Ampul No.	BAL mg.	TSH mg.	BAL + TSH	TSH	Ether Phase	BAL Phase	K
1	2431.6	21.1	0.988	0.127	0.592	10.1	.0586
2	2487.0	64.1	1.223	0.377	1.758	29.6	.0594
3	2443.9	122.9	1.529	0.703	3.277	56.5	.0580
4	2545.2	236.8	1.945	1.151	5.366	101.3	.0529
5	2535.4	329.5	2.217	1.449	6.755	137.7	.0489
6	Pure BAL	.....	0.868	0.000	...	...	Av. (1–4)
7	.....	Pure TSH	10.119	10.119	47.175	...	.0572

Approximately 2-ml. volumes of BAL were weighed into glass ampuls. The TSH, in varying amounts, was then weighed into the ampuls to give the desired concentrations. Exactly 2 ml. of petroleum ether was pipetted into each ampul and the ampuls quickly sealed with an oxygen-gas flame and shaken for 3 hours in a thermostated water bath at 24.5°. After removing each ampul from the bath, a 1-ml. sample of the petroleum ether phase was withdrawn as quickly as possible and pipetted into an excess of 0.1 *N* iodine; back titration was carried out with 0.1 *N* thiosulfate solution.

RESULTS

The data are presented in Table I. Columns *2* and *3* represent the quantities of BAL and TSH weighed into each ampul. The iodine titer of the petroleum ether phase, representing the sum of BAL and TSH, is given in column *4*. The iodine titer due to the TSH content of each ampul was calculated as follows. The iodine titer of the petroleum ether extract of the pure BAL (ampul 6) was multiplied by the mole fraction of BAL in the BAL phase of each ampul; the resultant corrected iodine titer due to the BAL in the petroleum ether extract of each ampul was subtracted from the corresponding titer of column *4* to give column *5*. The TSH content of the petroleum ether phase in mg./ml. is given in column *6*. The amounts of BAL and TSH in the BAL phase were then recalculated for the amount removed by extraction, and the concentration of TSH in the BAL phase is given in column *7*. The partition coefficient in column *8* is the mg. TSH per ml. petroleum phase divided by mg. TSH per ml. BAL phase. The average partition coefficient obtained, $K = 0.0572$, may be used in calculations applicable to the purification of BAL contaminated with trithiol.

The feasibility of the extraction method was verified by the multiple extraction of a typical BAL sample. A volume of petroleum ether equal to that of the BAL was used for each successive extraction step. The fraction of trithiol originally present which remains in the BAL after n extractions under these conditions is $(1 + K)^{-n}$. Therefore, 41 extractions would be expected to result in a 10-fold reduction in trithiol concentration. This was found to be the case experimentally. The percentage loss of BAL into the petroleum ether equals 0.435 × (number of volumes of petroleum ether used in the extraction). Except for convenience, little is gained in the way of efficiency by use of continuous extraction against a multiple extraction performed in this way.

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Identification of Some Barbiturates in Blood by Use of Thin-Layer Chromatography

By JOHN A. PETZOLD, WALTER J. R. CAMP, and ERNST R. KIRCH

A simple, rapid method for the identification of mixtures of barbiturates when present in blood is described. This procedure is especially useful in differentiating between amobarbital and pentobarbital.

THE PROCEDURES involving the identification of certain barbiturates leave much to be desired. This is particularly true when it is necessary to establish the presence of barbiturates in the blood and urine as the agents causing comas in hospital patients or deaths in medical-legal cases.

Two popular approaches of identification using paper seem to be those similar to Algeri, *et al.* (1), and Stevens (2), involving either the treatment of the paper chromatogram after elution of the barbiturate or treatment of the barbiturate before spotting in order to differentiate between saturated and unsaturated groupings attached to the pyrimidine ring. Among the many investigators using thin-layer chromatography, Frahm, *et al.* (3), using piperidine: petroleum ether as the mobile phase, and Eberhardt, *et al.* (4), using isopropanol: ammonia: chloroform as the mobile phase, have reported success in the separation of several barbiturates.

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TABLE I.—R_f VALUES OBTAINED USING STANDARD STOCK SOLUTIONS OF BARBITURATES[a]

Barbiturate(s)[b] Used	Untreated	Treated with 4N H_2SO_4
A	0.75	0.29
B	0.74	0.74
C	0.74	0.40
D	0.51	0.51
E	0.62	0
A + B	0.74	0.29 + 0.74
A + C	0.74	0.29 + 0.40
B + C	0.74	0.40 + 0.74
A + B + C	0.75	0.29 + 0.40 + 0.74
D + E	0.52 + 0.61	0.51
A + B + C + D + E	0.51 + 0.62 + 0.74	0.29 + 0.41 + 0.52 + 0.74

[a] Rf values given here represent the average of four determinations. [b] Code to barbiturate(s) used: (A) secobarbital, (B) amobarbital, (C) pentobarbital, (D) phenobarbital, (E) diallylbarbituric acid.

TABLE II.—R_f VALUES OF BARBITURATES EXTRACTED FROM BLOOD[a]

Sample No.	Barbiturate(s)[b] Present	Untreated	Treated with 4N H_2SO_4
1	A + B	0.75	0.28 + 0.74
2	A + D + E	0.52 + 0.62 + 0.74	0.27 + 0.41
3	C + E	0.62 + 0.74	0.40
4	A + B + D + E	0.52 + 0.62 + 0.75	0.29 + 0.51 + 0.74
5	A	0.75	0.29
6	A + B + D	0.51 + 0.74	0.30 + 0.51 + 0.74
7	A + C + D	0.51 + 0.74	0.29 + 0.40 + 0.52
8	A + B + C + D	0.50 + 0.75	0.28 + 0.40 + 0.51 + 0.74
9	C	0.74	0.40
10	A + B + C + D + E	0.50 + 0.62 + 0.75	0.27 + 0.39 + 0.50 + 0.74

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identification of five barbiturates more commonly encountered either alone or in a mixture during the toxicological studies in the Coroner's Laboratories of Cook County, Ill. The procedure is based on the differences in the R_f values obtained after treatment of the barbiturates with 4N H_2SO_4.

EXPERIMENTAL

Apparatus.—The apparatus used was essentially the one designed by Stahl (5), using 8 × 8-in. glass plates.

Adsorbent.—Silica gel G (Merck) was used.

Mobile Phase.—Acetone:*n*-butyl alcohol:ammonium hydroxide, concentration 9:9:2 was employed.

Reagents.—Silver acetate, A.R., 1% aqueous solution; diphenylcarbazone, A.R., 0.1% in 95%; ethyl alcohol; sulfuric acid, 4*N*; chloroform, reagent grade; sodium hydroxide, A.R., 0.45*N*; ammonium chloride, A.R., approximately 16%; Florisil, 60/100 mesh were employed.

Standard Solution of Barbiturates.—The following barbiturates were used in an alcoholic solution of a concentration of 2 mcg./μl.: secobarbital U.S.P., diallylbarbituric acid N.F., phenobarbital U.S.P., amobarbital U.S.P., pentobarbital U.S.P.

PROCEDURE

Each plate was covered to a thickness of about 250 μ with a paste consisting of 4 Gm. silica gel in 12 ml. distilled water. Precautions were taken to prevent air bubbles. The chromatoplates were dried in air for 15 minutes followed by a drying period of 30 minutes at 105°.

The plates while still hot were spotted in duplicate with 3 μl. of the alcoholic solution of the barbiturates. Immediately after application of the barbiturates, one spot of each duplicate was treated with 5 μl. of 4N H_2SO_4, and the chromatoplates were heated in an oven at a temperature of 125° for 1 hour. After cooling in air to room temperature, the chromatoplates were placed into a chamber containing the mobile phase. The solvent front ascended to the proper height within 1 hour. After drying at room temperature in a stream of air for 15 minutes, the barbiturates were developed as purple-blue spots using the standard method of spraying with silver acetate followed by the diphenycarbazone (1).

Table I shows the typical results obtained using the standard barbiturate stock solutions.

Extraction and Identification from Blood.—Five milliliters of blood containing barbiturate(s) were extracted with 50 ml. of chloroform and the extract evaporated to dryness in a stream of air. The residue was taken up in 10 ml. of chloroform, passed through a Florisil column, and the barbiturate(s) eluted with 10% methyl alcohol in chloroform as described by Stokes, *et al.* (6). The methanol-chloroform eluate was evaporated to dryness in a stream of air and taken up in 100 μl. of ethanol. Six to twelve microliters of the alcoholic extract was spotted on a silica gel chromatoplate and the barbiturates identified as described above.

When quantitative as well as qualitative results were desired the 0.45*N* NaOH extract obtained in the method of Stokes, *et al.* (6), was acidified to a pH of 2–3 and extracted with 50 ml. of chloroform. The residue left after evaporation to dryness in a stream of air was taken up in 100 μl. of ethanol and treated as described above.

Urine when extracted and purified as described by Stokes, *et al.* (6), produced comparable results.

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Effect of Inorganic Cation on the Fecal-Hydrating Action of Dioctylsulfosuccinate in Rats

By P. M. LISH and G. P. CASTEN

The calcium and sodium salt forms of dioctylsulfosuccinate were tested in rats for ability to prevent dehydration of the feces. The drugs were equally potent on a weight basis indicating that potency was directly related to the diester content of the materials and independent of the inorganic cation.

SODIUM DIOCTYLSULFOSUCCINATE[1] is now widely used in medicine as a fecal-softening agent. Recently a calcium salt of dioctylsulfosuccinate was marketed for the same purpose. The calcium salt is claimed to be twice as active as the sodium salt. The effectiveness of the sodium salt as a fecal softener in rats and mice was described recently by Lish and Dungan (1). The objective of the present study was to determine the influence of the cation on the fecal-hydrating action of dioctylsulfosuccinate in rats.

METHOD

The test materials were compared for ability to produce fecal-hydration following oral administration to rats. The technique used was adapted from Miller (2) with modifications essentially as described previously by Lish and Dungan (1). Minor deviations from the latter description were the use of ten animals per dosage level and restriction of observation periods to the 1, 2, and 4-hour intervals, post administration of the test agent. Rats were designated *positive* or *negative* responders depending on whether the water content of the dropped stools was sufficiently high to stain the absorbent paper on which they fell. The method of Litchfield and Wilcoxon (3) was used to evaluate the all or none type data.

Sodium content of sodium dioctylsulfosuccinate ($C_{20}H_{37}O_7SNa$; formula wt. 444.6) was 5.1%. Calcium content of calcium dioctylsulfosuccinate ($C_{20}H_{37}O_7S$ 1/2Ca; formula wt. 441.6) was 4.5%. All dosages were expressed as weight of the salt.

RESULTS

Results are shown in Table I. Data obtained at the 4-hour period post-drug dosage are presented graphically in Fig. 1. Dosages responsible for wet feces in 50% of the rats (ED_{50}) were 123.9 mg./Kg., and 110.8 mg./Kg., for the sodium and calcium salts, respectively. Statistical treatment revealed no significant difference in potency.

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[1] Marketed as Colace by Mead Johnson and Co., Evansville, Ind.

TABLE I.—EFFECT OF TWO SALT FORMS OF DIOCTYLSULFOSUCCINATE ON THE WATER CONTENT OF RAT FECES

Test Material		Positive Response/ Total Rats, Post-Drug Administration, hr.		
Name	P.O. Dosage, mg./Kg.	1	2	4
Sodium dioctyl-sulfosuccinate	70	0/10	0/10	1/10
	100	0/10	0/10	3/10
	150	6/10	7/10	8/10
	225	8/10	8/10	8/10
Calcium dioctyl-sulfosuccinate	70	1/10	1/10	2/10
	100	1/10	2/10	6/10
	150	4/10	6/10	6/10
	225	5/10	7/10	8/10

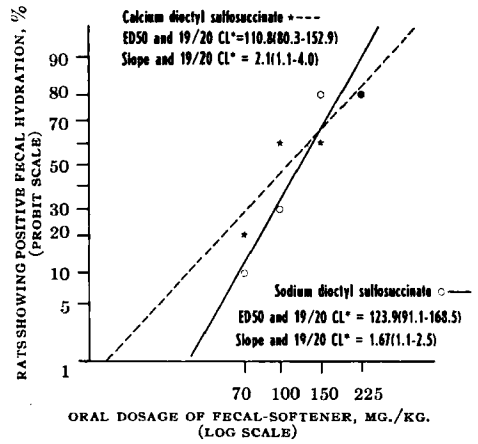

Fig. 1.—Log dose–probit relationships of the fecal-hydrating action of two salt forms of dioctylsulfosuccinate in the rat. Slope values, ED_{50} values, and $^{19}/_{20}$ confidence limits (C.L.*) according to Litchfield and Wilcoxon (3).

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Gas Chromatographic Separation of Amines by Special Selectivity

By J. S. SAWARDEKER and J. L. LACH

This study deals with the separation of several aliphatic amines by the use of metal complexation in GLC.

THE USE OF complex formation in the various branches of theoretical and applied chemistry and allied fields is now generally recognized. However, the use of complex formation, although highly promising, has received little attention in gas chromatography. Bradford (1), in his gas chromatographic studies employing solid silver nitrate in glycol, achieved a selective retardation of unsaturated hydrocarbons demonstrating interaction between vapor and metal atoms. Barber (2) reported marked increase in retention times of many individual compounds using molten stearates of metals, Mn, Co, Zn, Cu, and Ni as column liquids against retention times on apiezon L. Separation of amines in general has constituted a difficult task. Recently Sze (3) achieved separation of a mixture of ten amines using tetrahydroxyethylethylenediamine and tetraethylenepentamine as partition liquids in GLC. They report that the results are promising but not satisfactory.

EXPERIMENTAL

Equipment.—F & M 500 gas chromatograph with thermal conductivity cell and equipped with Minneapolis Honeywell recorder y143x(58).

Procedure and Results.—Two columns were prepared from 1/4-in. O.D. copper tubing. In column one, the stationary phase Gas Chrom P was coated with 2% SE-30 from a solution in methylene chloride, the solvent being removed by rotary vacuum evaporator. In the second, the stationary phase (Gas Chrom P) was coated with 2% SE-30 as above and then impregnated with 4% silver nitrate. A mixture containing triethylamine (b.p. 89.5°), diethylamine (b.p. 55.5°), and *n*-butylamine (b.p. 77.8°) in the proportion 1:2:1, respectively, was investigated using the two columns. Helium was employed as the carrier gas. The chromatograms are illustrated in Figs. 1 and 2. For the column containing SE-30 only, the mixture emerged as a single peak with isothermal operation at 50°. In the second column containing SE-30 and silver nitrate temperature programming from 50 to 175° was necessary for complete separation, since isothermal operation at 50° was ineffective. Apparently no decomposition was detected on the chromatogram. Isothermal operation at higher temperature gave poor results. Interestingly enough, it was found that the amines were well retained and the order of retention for the amines was 1° > 2° > 3°. Figure 3 shows the chromatogram of a mixture of triethylamine, diethylamine, and *n*-butylamine in the proportion 1:1:2, respectively, illustrating the separation achieved which may be quantitative. However, no attempt was made to study the separation quantitatively.

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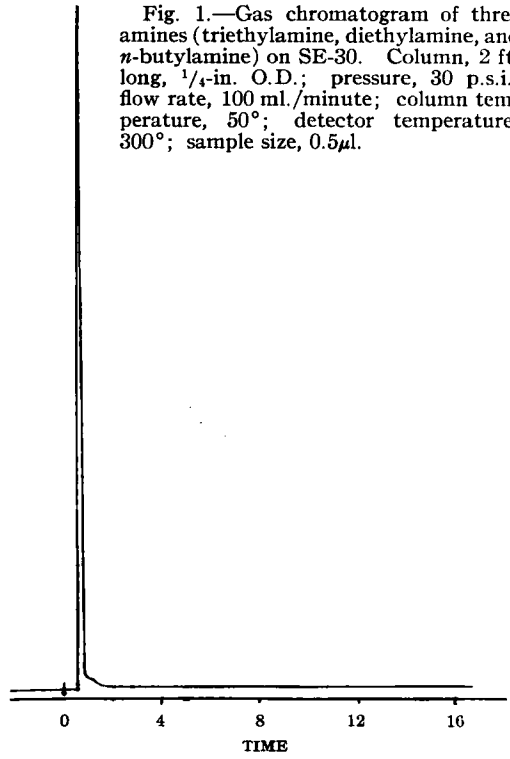

Fig. 1.—Gas chromatogram of three amines (triethylamine, diethylamine, and *n*-butylamine) on SE-30. Column, 2 ft. long, 1/4-in. O.D.; pressure, 30 p.s.i.; flow rate, 100 ml./minute; column temperature, 50°; detector temperature, 300°; sample size, 0.5 µl.

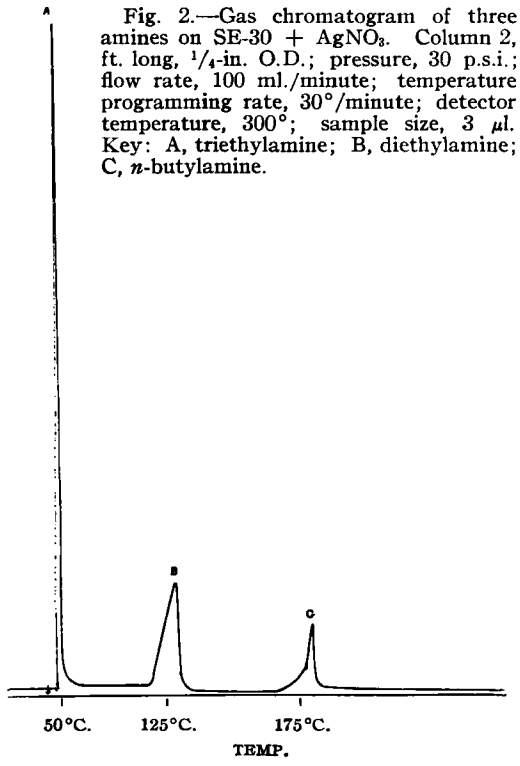

Fig. 2.—Gas chromatogram of three amines on SE-30 + $AgNO_3$. Column 2, ft. long, 1/4-in. O.D.; pressure, 30 p.s.i.; flow rate, 100 ml./minute; temperature programming rate, 30°/minute; detector temperature, 300°; sample size, 3 µl. Key: A, triethylamine; B, diethylamine; C, *n*-butylamine.

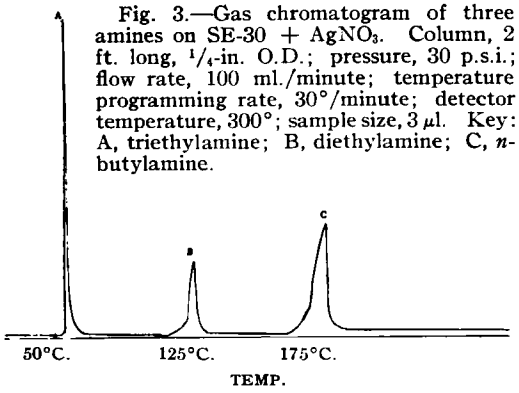

Fig. 3.—Gas chromatogram of three amines on SE-30 + $AgNO_3$. Column, 2 ft. long, 1/4-in. O.D.; pressure, 30 p.s.i.; flow rate, 100 ml./minute; temperature programming rate, 30°/minute; detector temperature, 300°; sample size, 3 μl. Key: A, triethylamine; B, diethylamine; C, *n*-butylamine.

DISCUSSION

It is evident from the chromatograms that the three amines pass through the various types of chromatographic columns at substantially different rates which are not governed by their boiling points. Silver nitrate seems to exhibit dramatic selectivity for these amines. It is difficult to state the exact nature of forces involved in the separation. However, in view of the fact that silver ion is capable of forming chelates, the strong retention behavior observed is indicative of chelate formation between the metals and the amines. It is of interest that a significant rise in temperature had to be employed to elute the 2° and 1° amines. This is consistent with the tendency for coordination increases with decreasing substitution $RNH_2 > R_2NH > R_3N$. As further evidence supporting complex formation, we observed that ethylenediamine, which theoretically is capable of forming a stronger chelate with silver, is so strongly retained that it could not be eluted even at 200°. Elution at higher temperatures was not attempted because of possible thermal decomposition of the silver nitrate. However, the use of silver bromide in place of the silver nitrate did permit use of higher temperature. In this silver bromide column ethylenediamine did emerge as a peak at a temperature of 250°.

It is of interest that the column length for this study was only 2 ft. Increasing the column length was not advantageous, and shortening it did not give good results. The method appears to be highly promising for separation of related compounds. Further studies are in progress; a complete report will be published at a later date.

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Synthesis of 5,5-Dimethyl-2,4-oxazolidinedione-2-C^{14} (DMO-2-C^{14})

By THOMAS C. BUTLER and JACK D. DAVIDSON

DMO-2-C^{14} was synthesized for use in measurements of the pH of intracellular water. It was prepared by condensing urea-C^{14} with n-butyl 2-methyllactate in the presence of sodium butylate in butanol solution. The yield of DMO-2-C^{14} from 1 mmole of urea-C^{14} was 58 per cent, and the product was radiochemically homogeneous as demonstrated by paper chromatography. The ionization exponent was determined by a system of solvent partitioning. A method is described for determination of DMO-2-C^{14} in biological samples.

THE MEASUREMENT of the pH of the water inside living cells is a difficult problem that has been approached in many ways (1). Of the various methods used, that based on the measurement of the intracellular and extracellular concentrations of a weak organic acid or base is probably most widely applicable and least subject to theoretical objections. Until recently, nearly all of the work based on this principle has employed carbon dioxide as the indicator compound. In 1959, Waddell and Butler (2) suggested that the weak acid, 5,5-dimethyl-2,4-oxazolidinedione (DMO), has the attributes desirable in a compound for the measurement of intracellular pH and should have advantages over carbon dioxide for that purpose. Using ultraviolet spectrophotometric methods for the analytical determinations of DMO in plasma and tissue, they employed the distribution of DMO for the calculation of the intracellular pH of dog muscle. Subsequently, DMO has been used by other workers in a number of different investigations of intracellular pH.

For an *in vitro* study of the intracellular pH of tumor cells, it became evident that the relatively large amount of DMO required in a sample for spectrophotometric measurement would impose undesirable restrictions on the design and scope of the experiments. A more sensitive method of measurement, as would be provided by the use of radioactive DMO, would avoid these restrictions. The synthesis of DMO-2-C^{14} was accordingly carried out. In the course of the subsequent study of tumor cells, experience with the use of DMO-2-C^{14} (in conjunction with inulin-carboxyl-C^{14} for measurement of extracellular water) has confirmed the expected advantages. Very small samples suffice, and the concentration of DMO added can be made low enough to cause no significant derangement of cellular function. Measurements of radioactivity

Received March 19, 1963, from the Department of Pharmacology, University of North Carolina, Chapel Hill.
Accepted for publication April 3, 1963.
This work was initiated at the Clinical Pharmacology and Experimental Therapeutics Section, General Medicine Branch, National Cancer Institute, Bethesda, Md., where Jack D. Davidson is a senior investigator and Thomas C. Butler was a visiting scientist.

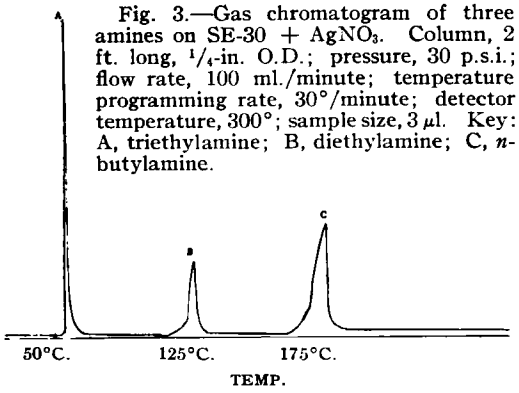

Fig. 3.—Gas chromatogram of three amines on SE-30 + $AgNO_3$. Column, 2 ft. long, 1/4-in. O.D.; pressure, 30 p.s.i.; flow rate, 100 ml./minute; temperature programming rate, 30°/minute; detector temperature, 300°; sample size, 3 μl. Key: A, triethylamine; B, diethylamine; C, *n*-butylamine.

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can be performed with greater rapidity and probably with greater accuracy than can the corresponding spectrophotometric analyses.

EXPERIMENTAL

DMO was synthesized by a modification of the method of Stoughton (3). Urea-C^{14} was condensed with an ester of 2-methyllactic acid in the presence of sodium butylate with the production of DMO and ammonia.

The *n*-butanol was dried over anhydrous potassium carbonate. The urea was desiccated. All glassware used prior to the addition of water was oven dried shortly before use.

A lump of clean sodium weighing somewhat more than 500 mg. was dissolved in 20 ml. of *n*-butanol. After titration, the solution was adjusted to contain 1 mmole of sodium per ml. In a 15 × 150-mm. glass-stoppered test tube 60 mg. (1 mmole) of urea-C^{14} of specific activity 1 mc./mmole was dissolved in 1.5 ml. of *n*-butanol with the use of heat. To this solution were added 1.0 ml. of the sodium butylate solution (1 mmole) and 0.17 ml. of *n*-butyl 2-methyllactate (1 mmole). The loosely stoppered tube was heated in an oven at 100° for 1 hour. It was then cooled in an ice bath, and 2.5 ml. of water was added. The tube was shaken and the butanol layer was transferred by pipet to another glass-stoppered tube, in which it was shaken with 2.5 ml. of 1 *N* sodium hydroxide. The butanol was discarded. The two aqueous extracts were combined in one tube and washed with two 5-ml. portions of ethyl ether, which were discarded. The aqueous solution was acidified with 1 ml. of concentrated hydrochloric acid and washed with 5 ml. of 2,2,4-trimethylpentane, which was discarded. The aqueous solution was then extracted with three 5-ml. portions of ethyl acetate, which were combined in a glass-stoppered tube. The ethyl acetate was washed with 2 ml. of water, which was discarded. It was then evaporated in the tube by drawing over a stream of air with an impinger (4). The residue, which at first was a colorless liquid, gradually became an almost completely crystalline white mass. After desiccation, the crude product consisted of 80% DMO by weight. It contained no radioactive constituent other than DMO and no nonradioactive impurity that interferes with its use. Further purification, with attendant losses, has accordingly not been carried out. The product has been kept in methanol solution, and portions of this solution have been evaporated for preparation of aqueous solutions for use in the biological experiments.

Radiochemical homogeneity was demonstrated by paper chromatography. A small volume of the methanol solution was spotted on Whatman No. 2 paper. A descending system was used in which 1,2-dichloroethane was the moving phase and water the stationary phase. The chromatogram was scanned with a Scanogram II automatic windowless paper chromatogram scanner with associated ratemeter and recorder. No radioactivity could be detected outside of a single spot with R_f 0.63. In one chromatogram 20 mcg. of authentic unlabeled DMO was spotted on a track adjacent to DMO-2-C^{14}. The nonradioactive track was cut into strips 2.5 cm. long, which were eluted with a buffer of pH 9 for study of ultraviolet absorption. The ultraviolet absorbing material was located at a position on the chromatogram identical to that of the radioactivity. DMO-2-C^{14} that has been in methanol solution for 3 years has been shown by paper chromatography still to be radiochemically homogeneous.

An aliquot of 1/100 of the total methanol solution was evaporated for preparation of an aqueous solution that was used to provide still smaller fractions of the total for measurement of the yield in terms of ultraviolet absorbing material and in terms of radioactivity. A buffer of pH 9 containing 1/10,000 of the total yield per ml. had an ultraviolet absorption spectrum identical to that of the ionic species of authentic DMO, with an absorption maximum at 208 mμ. From comparison with standards this solution was calculated to have a concentration of 7.5 mcg./ml. The total yield is thus calculated to be 75 mg. (58%). The radioactivity of a sample of 1/10,000 of the total yield was measured by liquid scintillation counting in comparison with a benzoic acid-C^{14} standard. The activity of the sample was 0.054 μc. The total C^{14} yield would be 540 μc., corresponding to a specific activity of 0.93 mc./mmole. Owing to uncertainties in the accuracy of the C^{14} assays, the spectrophotometric estimate of yield is the more accurate, but the agreement between ultraviolet absorption and radioactivity measurements is within the expected experimental error.

The ionization exponent of DMO-2-C^{14} was determined by partitioning a measured amount of the compound between 50% ethyl acetate–50% toluene (v/v) and several buffers of different pH values and of ionic strength of 0.16. The partitioning was carried out at 37°. Radioactivity in the upper phase was measured by liquid scintillation counting by the procedure described below for the determination of DMO-2-C^{14}. The partition coefficients were calculated from these measurements. The value of pK′ was calculated from the theoretical relationship between partition coefficient and pH of the aqueous phase (5). The value so calculated did not differ by as much as the experimental error from that of 6.13 as previously measured spectrophotometrically for the unlabeled compound (2). The value of 6.13 has accordingly been used in the calculation of intracellular pH.

The following method has been found suitable for the determination of DMO-2-C^{14} in biological materials. About 100 mg. of a liquid sample or of a preparation of homogenized cells is weighed in a tared glass-stoppered test tube. One milliliter of 5 *M* monobasic sodium phosphate plus 5 ml. of 50% ethyl acetate–50% toluene (v/v) are then added to the tube. After the tube is shaken and centrifuged, 4 ml. of the upper phase is transferred to a counting vial. To the vial is then added 14 ml. of a solution of the following composition: toluene 93% (v/v), methanol 7% (v/v), and 2,5-diphenyloxazole 0.4% (w/v). Radioactivity is measured by scintillation counting. This method is applicable to samples containing inulin-carboxyl-C^{14} and DMO-2-C^{14}, since the former compound is not extracted into the organic solvent.

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Reinvestigation of the Biological Activities of Diacetylapomorphine and "Triacetylapomorphine"

By JOSEPH G. CANNON, JEROME F. HENSIAK, and ALLAN M. BURKMAN

Diacetylapomorphine has been shown to possess emetic activity nearly identical to that of apomorphine itself, corroborating findings of some earlier workers and contradicting certain others. "Triacetylapomorphine" has been demonstrated to be completely nonemetic (in agreement with the original literature report) and to possess moderate hypotensive properties.

DIACETYLAPOMORPHINE and "triacetylapomorphine" were first prepared by Tiffeneau and Porcher (1) in 1915. "Triacetylapomorphine," an amide resulting from ring cleavage of diacetylapomorphine, was reported as being devoid of emetic activity.[1] In a series of papers, Tiffeneau first reported that diacetylapomorphine was equally as potent an emetic as apomorphine (2); later, that it was twice as potent (3). Tiffeneau's explanation for this high emetic activity of diacetylapomorphine was that it is rapidly hydrolyzed in the body to free apomorphine. However, the experiments of Eddy (4) tend to contradict this hypothesis; this worker demonstrated that, in compounds related to morphine, masking of hydroxyls by alkyl or acetyl functions eliminated central emetic activity.[2] Of particular significance is 3,4-dihydroxyphenanthrene, which is structurally similar to apomorphine and is a moderately potent emetic; diacetylation renders this molecule nonemetic.[2] Since the work of Tiffeneau is rather old, and since his various publications contradict Eddy's reports and each other, it seemed advisable to reinvestigate the pharmacological properties of the acetylation products of apomorphine.

"Triacetylapomorphine" was prepared by heating apomorphine hydrochloride in acetic anhydride according to the method of Tiffeneau (1). It displayed no emetic activity when administered subcutaneously to dogs in a dose range up to 100 times the dependable emetic dose of apomorphine. It did not elicit the characteristic pecking syndrome in pigeons (5), and it exhibited no antiacetylcholinesterase activity. However, it showed moderate hypotensive activity.

Diacetylapomorphine was prepared in 10% yield by treating apomorphine hydrochloride with acetic anhydride and pyridine at room temperature according to the method of Small (6), using Tiffeneau's procedure (1) for recrystallization. This compound appeared to be identical to apomorphine in regard to minimal emetic dose and time of onset of action, although the severity of vomiting is of a lower order. It is a potent pecking syndrome stimulant in the pigeon.

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[1] The abstract of *Reference* 2, as reported in *Chem. Abstr.*, 8, 3191(1914), incorrectly states that "triacetylapomorphine" is identical to apomorphine in its physiological properties.

[2] While the acetylated and alkylated compounds often induce symptoms of nausea in the cat (as indicated by excessive salivation and licking), they are considered here as being nonemetic since they did not cause actual vomiting.

TABLE I.—RESULTS OF BLOOD PRESSURE LOWERING EXPERIMENT

Min. After Injection	B.p., mm.	Respirations/ Min.
0	80	21
0.5	80	20
1	82	23
3	66	28
5	45	25
10	37	25
15	30	22
20	32	24
30	29	24

EXPERIMENTAL

"Triacetylapomorphine" was triturated in a few drops of polysorbate 80[3] and was suspended in 50% propylene glycol solution. For the emesis test, 0.1 to 10 mg./Kg. was administered subcutaneously to six dogs, from whom food had been withheld for 16 hours; however, 15 minutes prior to the injection, the dogs were given about 0.25 lb. of food, an amount insufficient to allay hunger, but enough to provide some bulk in the stomach in the event that vomiting were elicited. During an 8-hour period, there was no evidence of emesis or nausea; that is, there was no salivation, and appetite remained.

For the blood pressure lowering experiment, 4 mg./Kg. of "triacetylapomorphine" was administered intravenously to a 10-Kg. dog, anesthetized with sodium pentobarbital and previously tested for tone and autonomic dominance of peripheral blood vessels in the following manner: Carotid occlusion gave a substantial rise in blood pressure; intravenous injection of 1.5 mcg./Kg. of epinephrine hydrochloride brought about a 25-mm. rise in blood pressure and respiratory depression; intravenous injection of 0.5 mcg./Kg. of methacholine chloride resulted in a 35-mm. drop in blood pressure and mild hyperpnea. The results of the experiment are recorded in Table I.

Carotid occlusion reflex was tested and epinephrine injected at intervals; carotid reflex was depressed but not abolished, and epinephrine response was unaltered.

Diacetylapomorphine was dissolved in a small amount of 0.1 *N* HCl, and was administered in solution, buffered to pH 5.5 with MacIlvain's phosphate-citrate buffer. Four dogs received doses of 0.1 to 0.2 mg./Kg. subcutaneously. For comparison, two dogs received apomorphine hydrochloride, 0.1 and 0.2 mg./Kg., respectively. Emesis occurred within 5 minutes in all cases.

[3] Marketed as Tween 80 by Atlas Powder Co., Wilmington, Del.

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Communications

Effect of Some Perfume Oils on Dehydrogenases in *Escherichia coli*

Sir:

Since the discovery that perfume oils inhibit the growth of bacteria (1, 2) we have investigated the antimicrobial properties of many such oils further (3, 4). The mode of action by which bacteria are killed by perfume oils remains unknown but Gal'perin and Dunaeva (5, 6) have reported that essential oils affect dehydrogenases in paramecia and helminths. Since essential oils are commonly used in perfume oil formulas it was thought that bacterial dehydrogenases might be affected by perfume oils.

We selected as the test organism *Escherichia coli* ATCC 11229. The bacteria were harvested after 24 hours from nutrient agar slants and washed three times with sterile physiological saline by centrifugation. A 2-ml. quantity of washed packed cells was added to 3 ml. of 1:1000 perfume oil solution (1% alcoholic). After 30 minutes of contact the cells were removed by centrifugation and washed three times with sterile saline and suspended in saline to 300 Klett units in the Klett-Summerson photoelectric colorimeter using the No. 42 filter with a spectral range of 400 to 465 mμ. This treated standard cell suspension was used for all subsequent experiments.

The dehydrogenase activity was determined by the Thunberg technique (7). The substrates used were glucose, succinic acid, and malic acid. The color intensity of the methylene blue in the Thunberg tubes was recorded every 4 minutes for a total of 32 minutes. All experiments were run at least in duplicate with controls and were replicated several days later.

Perfume oil of bouquet No. 821, sweet grass, lilac water, and bouquet No. 21 completely inhibited glucose, succinic acid, and malic acid dehydrogenase. Plate counts of the standard cell suspension showed no reduction in cell number compared to controls. However, after 2-hour exposure of the test organism to each perfume oil, plate counts revealed a 10 to 50% reduction depending upon the oil used. This finding suggests that perhaps the reduction of dehydrogenases precedes the death of the cell.

E. coli was exposed to perfume oil of neroli (artificial), evergreen bouquet, osheana, and chypre 66D at a concentration of 1:2000 in the same manner as above and dehydrogenase activity measured. It was found that oil of neroli and evergreen bouquet completely inhibited all tested dehydrogenases, while oil of osheana and chypre 66D caused little effect on glucose dehydrogenase, more than 90% inhibition of succinic dehydrogenase, and complete suppression of malic dehydrogenase.

Essential oils and their components are presently being investigated for their effects on bacterial dehydrogenases.

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The authors thank Colonel Joseph Baird Magnus of Magnus, Mabee and Reynard, Inc., New York, N. Y., for the perfume oils used in this investigation.

Studies on *Catharanthus lanceus* (*Vinca lancea*) I. Isolation of Leurosine, Perivine, and Yohimbine

Sir:

Demonstration of reproducible anticancer activity by extracts from *Catharanthus roseus* (*Vinca rosea*) against the P-1534 leukemia in DBA/2 mice prompted Svoboda, *et al.*, to initiate intensive phytochemical studies on this plant to establish the nature of the substance(s) responsible for this activity. These investigators have reported on the isolation of more than 40 alkaloids from *C. roseus*, including four with oncolytic activity: vincaleukoblastine (1),[1] leurosine (3), leurocristine, and leurosidine (4).

Two of these alkaloids are now available for use in the treatment of Hodgkin's disease,[2] choriocarcinoma,[2] and acute leukemia in children.[3]

We have initiated studies on leaf material from a related plant, *Catharanthus lanceus*,[4] using the alkaloid fractionation scheme reported by Svoboda, *et al.* (5). Alumina chromatography of the (*A*) fraction has yielded leurosine and perivine, previously reported only from *C. roseus* (3) in addition to yohimbine which has been reported from *C. lanceus* roots (6) and several other sources. The identity of these alkaloids was established by comparison of certain physical data to that of reference material. The data used were derived from infrared and ultraviolet absorption spectra, electrometric titrations, X-ray powder diffraction patterns, melting point and mixed melting point determinations, and thin-layer chromatographic studies. Details concerned with the isolation of these three alkaloids and others will be published at a later date.

It is also of special interest to communicate that leurosine, an alkaloid reported effective against the P-1534 leukemia in DBA/2 mice (7), was isolated in the course of our investigation from an alkaloid fraction which on replicate testing proved ineffective in producing a prolongation of life against this tumor system. This confirms a previous report by Svoboda indicating that alkaloids highly active against the P-1534 leukemia can be present in crude fractions which exhibit limited or negative antitumor activity under identical test conditions (4). Svoboda has advanced that this anomaly could be due to toxic inactive material being present in crude mixtures which also contain active anticancer alkaloids (4).

This confirmation of the isolation of an active oncolytic alkaloid from an inactive crude fraction, suggests that a reevaluation of current test methods used for the detection of anticancer activity in crude botanical extracts is necessary.

[1] First reported by Noble, Beer, and Cutts (2).

[2] Marketed as Velban (vinblastine sulfate) by Eli Lilly and Co., Indianapolis, Ind.

[3] Marketed as Oncovin (vincristine sulfate) by Eli Lilly and Co., Indianapolis, Ind.

[4] The *Catharanthus lanceus* (Boj. *ex* A. DC.) Pich. leaves used in this study were collected in Madagascar and supplied by the S. B. Penick Co., New York 8, N. Y. An authentic herbarium specimen of the material used in this study has been deposited at the above address.

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The Editor comments

GOVERNMENT'S ROLE IN RESEARCH

A special Committee has been created in the U. S. House of Representatives with the specific purpose of conducting an investigation into government spending and involvement in science, research, and technology. The Select Committee on Government Research under the chairmanship of Rep. Carl Elliott, was just concluding its first few days of hearings when the assassination of President Kennedy brought an abrupt interruption to the Committee's activities. However, the hearings are expected to resume this week.

The A.PH.A. is among those who have been asked to testify; in fact, its spokesman originally was scheduled to appear on the Tuesday morning following the ill-fated Friday in Dallas. It should be noted that the Elliott group plans a very broad spectrum investigation into all research activities in which the federal government participates. As such, the invited witnesses cover a broad range including many from aeronautics and rocketry. The preliminary calendar released by the Committee's staff listed the A.PH.A. as the only organization invited from the pharmaceutical field, and with the exception of the American Medical Association, the only non-government organization from the health field.

It is our opinion that the inquiry being conducted by this Committee can have a very healthy effect on all of our research efforts—private as well as government. Federal support for research and technology has increased at an unbelievable rate during the past two decades, from $74 million in 1940 to $12.2 billion in 1963. Considering the size of this expenditure, relatively little overall coordination or long range planning has been devoted to making sure that the most efficient and effective programs are being supported in an effort to reach goals which are laudable, but which at times are very general or ill-defined. Closer Congessional scrutiny should also serve to help avert repetition of those few cases of loosely managed funds which have recently come to the attention of Congress, and which have made virtually all federal research expenditures suspect in the eyes of some Congressmen.

Consequently, it appears to be a very appropriate time for stock-taking. We sincerely hope and expect that the inquiry will prove to be beneficial to the federal government, the project grantees, and the American taxpaying public.

Edward G. Feldmann

JOURNAL OF Pharmaceutical Sciences

December 1963 volume 52, number 12

Review Article

Preclinical Evaluation of Drugs for Evidence of Teratogenic Activity

By HAROLD M. PECK

"If certain congenital malformations can be attributed in certain animals to dietary deficiency, anoxia, cortisone, or genetic constellations, one must not conclude without further proof that comparable malformations in man are due to similar adverse conditions. Such premature conclusions, usually not drawn by the experimentor but by a reader whose imagination and beliefs exceed his knowledge, can create superstitions in modern garb. If such a reader is also a writer with access to medical or popular journals, his unfounded beliefs are carried to millions, whereby new superstitions are established. . . . A single observation of an abnormal child born to a mother who had been in an automobile accident, becomes, if reported in a popular magazine, psychologically, a hundred thousand observations which seem to establish a causal relationship between two events. The whispered word is powerful, but the written word endures." Joseph Warkany, National Foundation Conference on Congenital Malformations, 1959 (1).

THE CIBA FOUNDATION SYMPOSIUM on Congenital Malformations (2) and reviews by Wilson (3) and Giroud and Tuchmann-Duplesis (4) refer to many agents that have been shown to have teratogenic effects in animals. These vary from anticancer drugs to essential nutritional elements as shown by the following:

TERATOGENIC AGENTS FOR ANIMALS

Alkylating Compounds
- Chlorambucil
- Busulfan (human)

Cytotoxic or Antimitotic Agents
- Actinomycin D

Nutritional Factors

Deficiency
- Oxygen
- Riboflavin
- Pantothenic Acid
- Folic Acid
- Vitamin A

Antimetabolites
- 6-Aminonicotinamide
- Methotrexate

Physical
- X-ray (human)
- Nuclear
- Ultrasonics
- Trauma

Excess
- Vitamin A
- Carbon Dioxide

Infectious Agents
- Rubella (human)
- Influenza (?) (human)

Some drugs used to treat cancer are effective because of their depressant effects on rapidly proliferating cells and, therefore, may have an adverse effect on the rapidly growing animal or human embryo. Similarly, antimetabolites, by interfering with essential nutritional materials such as vitamins, could be expected to have teratogenic properties. The effect of German Measles, or Rubella, on the human fetus is well known, but the effect of other viral agents such as the influenza virus (5) has not been substantiated.

Some other well known agents have been

Received from the Merck Institute for Therapeutic Research, West Point, Pa.
Based, in part, upon a presentation to the Scientific Section, A.PH.A., Miami Beach meeting, May 1963.

shown to have teratogenic properties in animals but do not have any proven effect on the human fetus.

TERATOGENIC AGENTS FOR ANIMALS

Penicillin	Salicylates
Chlorpromazine	Trypan Blue
Prochlorpemazine	Galactose
Hypoglycemia Sulfonamides	Thalidomide (human)
Insulin	Pilocarpine
Chlortetracycline	Sulfanilamide

Seemingly, thalidomide is the outstanding exception. Other drugs, as an aftermath of the thalidomide incident, have been accused of being teratogenic for man, but a causative relationship has not been proven to the satisfaction of all. No doubt many other drugs will be accused of producing malformations in humans.

Warkany (6) stated the problem clearly when he said:

> "We should be very cautious in making general statements about these drugs. One could easily make a mistake and accuse a drug wrongly. The cases in which a drug has been proven to be teratogenic in humans are very few. Dose and time play a role, and probably also the genetic constitution. But, if we are not careful, we may hear very soon that aspirin causes malformations. This should not happen since our animal experiences are usually very different from therapeutic attempts in man."

Murphy (7) pointed out that in the embryo the outcome of a drug effect, under the conditions of a limited number of doses—

> "Depends also on the specific drug as well as the quality and density of events taking place within the embryo. . . . The choice of a mammal with a longer gestation time might be expected to accomplish a decrease in the density of events taking place during a given time period and, therefore, decrease the probability of a foetal abnormality from a single dose of a growth-inhibiting agent. On the other hand, choice of an animal with a much shorter period of gestation would accomplish the purpose of increasing the density of events occurring during a given period and increase the probability of abnormalities."

This would suggest that, of the laboratory animals commonly used for preclinical evaluations of safety, the mouse would be the ideal species for testing for the teratogenic properties of a drug and the dog would be least valuable.

However, the conditions employed in the more academic teratogenic studies may not apply to the preclinical evaluation of safety since the drug should be administered daily in reasonable, nonstressing doses.

The problem of evaluating the safety of a drug for the fetus is not new and is similar to that routinely faced by the toxicologist and the clinician when a new compound is evaluated for its safety for initial clinical trials. The nature of the toxicity of a drug for the animals may be well known and on the basis of this knowledge and the other biological properties of the agent, a scientific judgment must be made of the relative risk for its trial in man. In the same way, as Beyer (8) pointed out—

> "Since such a substantial list of important agents can elicit fetal anomalies under laboratory conditions, the relevance of such findings to the prognostication of similar clinical outcomes requires a broad basis for judgment. Depending on methodology and experimental design, an important teratogenic action of one compound could be missed and another might be indicted unjustly."

The toxicologic and teratogenic evaluations of a drug are interrelated and require the same general considerations of its chemical and biologic properties and proposed clinical use. It may be useful then to discuss briefly the principles of the toxicologic evaluation of a new drug since these principles will apply also to the teratogenic evaluation of that drug.

The toxicologic, or safety, evaluation of new drugs in animals encompasses a great deal more than the determination of acute lethal doses and the daily administration of relatively small doses which may provide a relative "margin of safety." It is important also to produce toxicity in subacute and chronic studies. As the complexity of the biologic properties of drugs increases and as our knowledge of the mechanism of action on these drugs increases, the testing for possible adverse side effects can expand also to provide greater assurance of safety and to alert the clinician to potential toxic effects in the human. Factors which should be considered in the safety evaluation of therapeutic agents are:

- Chemical structure and properties
- Route of administration
- Reversible alterations
  - Primary and secondary biologic attributes
  - Endocrinologic properties
  - Biochemical properties
  - Organ function
  - Normal body constituents
  - Anatomical changes
    - Gross and microscopic
    - Organ weights
- Metabolism of the drug
  - Degradation
  - Conjugation
  - Elimination

Clinical
Proposed use (disease, dose, duration of treatment, etc.)
Possible misuse

Thus, the safety evaluation of a drug may now include an overall consideration of its chemical properties, its pharmacologic, endocrinologic or biochemical attributes, its effects on normal body functions and normal body constituents, the manner in which the body excretes or degrades the agent, and the potential of the drug to produce reversible changes of body function or anatomical structures. Any of these properties might be a primary attribute of the drug and the activity necessary for its therapeutic use. In addition, the proposed clinical use, and possible misuse, of the agent must be considered. In general, the more serious the disease, the more risk is acceptable; conversely, if a disease is of minor consequence, the drug must have a large safety factor.

TYPES OF UNDESIRED EFFECTS

The types of undesired effects of a drug may be grouped into four general classes: (*a*) exaggerated primary attributes, (*b*) secondary attributes, (*c*) hypersensitivity, and (*d*) "true" toxicity.

The first class includes the undesired effects due to overdosage, or to an accentuation of the desired effects of the drug. Some individuals may be unusually sensitive to the drug so that an average therapeutic dose may become an overdose. Occasionally, the interaction of two or more simultaneously administered drugs may result in an accentuation of the biologic attributes of one of the drugs.

The second class of side effects includes those related to the secondary biologic attributes of the agent. The dryness of the mouth caused by anticholinergic agents, the nausea and respiratory depression caused by the narcotics, and the sedation produced by some antihistaminics are examples of these. Such side effects are usually reversible when the drug is withheld and are not necessarily detrimental to the health of the individual. Nevertheless, they are disturbing and, thus, there is always an effort on the part of the organic chemist and the pharmacologist to synthesize new compounds (molecular manipulation) to reduce the secondary attributes, and to increase, relatively, the effect of the primary attribute.

The third class, hypersensitivity, includes a large group of undesired side effects resulting from the sensitization of an individual to the drug. These side effects may be severe, as in anaphylaxis, or may be relatively minor, as in a skin rash. Unfortunately, there is no reliable methodology by which the incidence or type of hypersensitivity can be predicted by presently known animal experiments.

The most important class of side effects is that of the "true toxicity" of a drug. This toxicity may be unrelated apparently to any known pharmacodynamic activity of the drug and, therefore, may be unpredictable from the results of the pharmacological studies. As a general rule, the production of these important side effects requires daily administration of the drug and, thus, they are not disclosed in the short term pharmacologic studies where only one or two doses may be given. Indeed, several months of daily administration of large doses may be required to produce a toxic effect. A relationship between incidence of toxic effects and magnitude of dose is usually present, thus permitting an estimation of a "margin of safety." The "true toxicity" may result in reversible or irreversible lesions and may be detrimental to the health of the individual.

One of the functions of the pharmacologist in the safety evaluation of a new drug is to set the probable clinical dose and to predict the side effects of the first two classes. The function of the toxicologist is to demonstrate the hazards related to the first three classes and, of most importance, he must determine the potentialities of the drug with regard to the production of "true toxicity" and also must make an evaluation of its effect on reproduction.

To fulfill these functions, the toxicologist assumes that all agents are toxic if examined under the proper conditions using appropriate parameters of observation. The toxicity of a drug is examined by the acute administration of lethal doses and by the daily administration of moderate and large doses for short (subacute) and long (chronic) periods of time. He may use several routes of administration but the major route is that which is to be used clinically. Two or more species of animals may be used in both the long and short term studies since there may be species differences in sensitivity to the drug. The toxicity found in these studies may be biochemical, hematologic, endocrinologic, enzymatic, anatomic, etc., or any combination of these.

The pharmacodynamic activity of the drug may be such that the primary or secondary attributes limit the toxicologic investigations because sufficiently large doses cannot be used to produce the "true toxicity." If, for example, a

sedative action is sufficiently great, the animal cannot eat or drink properly and, since prolonged fasting or starvation can cause biochemical and anatomical alterations, it may be difficult to determine if starvation effects are related to starvation or to the "true toxicity" of the drug. In other words, in the safety evaluation of a drug, the toxicologist cannot permit primary or secondary drug effects to alter the animal sufficiently that the end results may be attributed incorrectly to a toxic effect of the agent, or, that a "true" toxic effect may be hidden by these indirect effects. In these cases, the toxicologist must limit the magnitude of the doses to levels that will permit a relatively normal daily activity of the animal.

TERATOGENIC STUDIES

All of this may appear to be far from the subject of evaluation of teratogenic effects of drugs. However, the preceding remarks provide a basis for the principles of the evaluation of the teratogenic properties of an agent. Factors such as anorexia, hormonal changes, or sedation, which can be primary attributes of drugs, may have an effect on reproduction or on the fetus and must be considered. It certainly would not be reasonable to give such large doses of an agent that anorexia would occur for long periods of time and then to expect to have a normal reproduction cycle and normal fetuses. Similarly, if one is examining the teratogenic effects of a barbiturate in a study which requires dosing prior to mating, the dosage must be sufficiently small, or temporarily stopped, so that sedation will not interfere with mating.

There are three major types of teratogenic studies that can be employed for safety evaluations of new drugs:

Established pregnancy
  Single dose
  Multiple dose
Single generation (breeding) study
  Drug administration prior to mating and throughout pregnancy
  Two or more litters by same female
Multiple generation studies
  Drug administration prior to mating and throughout pregnancy
  Young animals fed medicated diet after weaning and mated when mature
  Two or more generations produced

The first is the administration of drug to animals in which pregnancy has been established. Drug administration is initiated, usually several days after conception, and may be continued for several days or as long as desired. Such studies are of relatively short duration and are particularly useful in the larger animals. Here, there is no possibility that the drug will interfere with fertility, mating, or implantation of the ova. The study can be continued through delivery, lactation, and weaning. Single doses may be of no real value at an early stage of development of a new drug. Indeed, this type of study may be hazardous because a teratogenic effect of an agent may be limited to a very brief phase of embryonic or fetal development and thus missed if the single dose is given at the wrong time.

The second type of teratogenic study includes more than just testing for teratogenic effects and may be called a "single generation study" or "breeding study." In this experiment, both the male and female animals are given the drug daily for some period prior to mating (60 days has been suggested), and drug administration is continued throughout pregnancy and, if desired, until the pups are weaned. Holding the pups until weaning may reveal types of malformations which would not be evident if the animals were sacrificed and examined at birth. The breeder animals continue to receive drug and, after the first litter is weaned, should be remated to produce a second litter. In some instances, part of the mothers may be sacrificed just before delivery and the fetuses examined. If a normal incidence of pregnancy is not obtained, the treated female animals may be mated with control male animals and treated male animals with control female animals to determine if the fertility of one sex is impaired. The large number of parameters that can be evaluated point out the usefulness of this type of study. In addition, this reproduction study can be conducted as a part of the chronic toxicity studies. Thus, in single or multiple generation reproduction studies it is possible to evaluate

Fertility of male and female,
Implantation of the ova,
Development of the embryo and fetus,
Resorption,
Abortion,
Delivery,
Live births,
Size of litters,
Teratogenic effects,
Viability of the newborn,
Growth of young,
Quality of mother's milk (nutritional and toxicity).

A third type of reproduction study is the multigeneration experiment. Here a litter is produced, the pups are permitted to mature, and are mated to produce a new generation. Drug administration is started for the original parents, prior to

maturing, and continued for each generation until the desired number of generations have been obtained. This cycle can be repeated as frequently as desired, precautions being taken to prevent mating between litter mates. The parents of each generation may be discarded when the young are weaned. An evaluation of possible genetic abnormalities produced by an agent may be detected by this type of experiment as well as the parameters listed in the second type of study mentioned previously. These studies are necessarily of relatively long duration, depending upon the reproduction cycle of the species being used. The mouse is probably the best species for this type of test because of the relatively short reproduction cycle, rapid maturation, and the large number of animals that can be employed.

In all of these studies, the young animal can be examined in a number of ways. Gross examination of the fetus, or the newborn, clearing and staining of the fixed animal, X-ray studies, and autopsy examination permit an evaluation of teratogenicity. The specimen can be prepared for microscopic examination also. Enzyme studies can be made of the fetus or the newborn. Acute toxicity studies may be conducted on the control newborn or weaned animals to determine age differences of drug toxicity.

The use of an adequately large control group of male and female animals is a must for all teratogenic, reproduction, and routine toxicity studies. Incidental lesions occurring within animal species are so frequent that, unless there are enough controls, certain types of lesions (or fetal malformations) may be difficult to evaluate with regard to drug effect.

The conduction of these studies appears to be rather straightforward. However, because environmental factors may interfere with normal reproduction cycles in some animals, it is advisable to use quiet draft-free rooms with well regulated temperature and humidity. It is advisable also to have special personnel assigned to such studies so the animals will be accustomed to them and will not be disturbed at critical times such as delivery. Variations in litter sizes, fertility, and percentage pregnancies may complicate the interpretation of results. Thus, it may be wise to obtain a first litter without treatment, although this adds considerably to the duration of the experiment. It is also important to know, if possible, the normal incidence and types of malformations that occur in the species and strains of the animals being used.

A useful collateral study is the determination of the transfer of the drug across the placental membrane. Such a test might determine the best species to be used in a teratogenic evaluation of a drug, since the value of a teratogenic study is questionable if the drug is not transferred across the placenta to the fetus. It is reassuring to be able to give large doses of a drug to a pregnant animal without evidence of any effect on the reproduction cycle, or on the fetus, when it is known that large amounts of drug, or its metabolites, actually reach the fetus.

As indicated earlier, the dosage of the drug should be selected so that the secondary pharmacodynamic effects will not interfere with conception or nutrition (9) and activity of the mother. In addition, since some agents may interfere with normal biologic functions as a primary attribute, this type of effect on the fetus must be judged carefully. In other words, the doses selected should take into consideration the therapeutic and tolerated doses and not be so far above the therapeutic dose, or so close to the toxic dose, that the reproduction studies cannot be interpreted meaningfully. For example, by using a single maximally tolerated intraperitoneal dose of caffeine, Nishimura (10) obtained an incidence of 18 to 43% malformations in the mouse. Thus, there is a possibility that the occurrence of malformations at unreasonably high doses might result, unjustly, in the rejection of an otherwise valuable agent.

Other methods of teratogenic studies may not be applicable because the conditions of these tests are not comparable to the conditions obtained in the uterus of the intact mammal. Thus, the chicken embryo (11–14), the seaurchin (15, 16), the isolated fertilized rabbit egg (17), and the zebra fish egg (18, 19) may be valuable for certain aspects of teratogenic studies, but they lack the pharmacologic response, the metabolic alterations, and the elimination of the drug which may occur in the mammal. These studies appear to be most useful for studying the mechanism of action of known teratogenic agents, but their value for determining the teratogenic properties of unknown agents in the evaluation of safety has not been determined. There is too much chance of missing a critical period in a one or two dose experiment and thus a potent teratogenic agent might be accepted as safe. As these techniques accumulate, they may be found useful as collateral studies, but we should not, at the present time, accept them in place of the mouse, the rat, or the rabbit as the major tests for teratogenic effects of a drug. Nor should positive teratogenic effects in these

specialized tests be permitted to overweigh negative results in the mammal.

In conclusion, the evaluation of new drugs for possible teratogenic properties may be conducted as a part of the toxicologic evaluations of the same agents. The same considerations of the chemical and biological properties, the metabolic fate of a drug and its proposed clinical use, as applied to an evaluation of its toxicity, may be applied also to its teratogenic evaluation. Teratogenic studies of new agents should be conducted under reasonable nontoxic daily dosage schedules in the intact mammal to permit a more valid interpretation of the results than may be obtained from single dose experiments, or than may be obtained from the results of studies in the nonmammalian species.

At the present time, if new drugs can be shown to have teratogenic potentialities in animals, such findings should, at most, serve as a warning to clinicians—

> "If such (animal teratogenicity) became a legal barrier to human consumption, patients would be denied the benefits of some antibiotics, cortisone and even aspirin" (20).

SUMMARY

The evaluation of new drugs for possible teratogenic properties may be conducted as a part of the toxicologic evaluations of the same agents. The same considerations of the chemical and biological properties, the metabolic fate of a drug and its proposed clinical use, as applied to an evaluation of its toxicity, may be applied also to its teratogenic evaluation. Teratogenic studies of new agents should be conducted under reasonable nontoxic daily dosage schedules in the intact mammal to permit a more valid interpretation of the results than may be obtained from single dose experiments, or than may be obtained from the results of studies in the nonmammalian species.

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Biopharmacology of Anabolic Agents II

Radio-Phosphorus Uptake in Molars of Rats Fed Norethandrolone and Phosphorus-Enriched Caries-Test Diets

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No statistically significant difference existed between the radio-phosphorus content in the molar teeth of animals treated with norethandrolone and the respective control animals. In addition, radio-phosphorus levels in the molar teeth of norethandrolone-treated animals fed a high phosphorus diet equalled the radio-phosphorus level in those animals on a high phosphorus diet alone. These results were not effected by the age of the animal within the experimental period studied, nor was there a difference in these results upon comparison of first, second, and third molar teeth with their respective controls.

CONSIDERABLE experimental evidence concerning the cariostatic effect of phosphorus may be found in the literature. For example, a very pronounced cariostatic effect has been obtained with supplements of dibasic sodium phosphate (1–9). Previous experiments in our laboratories (10) indicated that when 2% disodium phosphate is added to a caries-test diet, Sprague-Dawley strain albino rats have less tooth decay in molar teeth than the respective controls.

The addition of norethandrolone to the caries-test diet fed Sprague-Dawley albino rats increased the incidence of molar tooth decay over that found in animals fed the basic caries-test diet alone. When 2% disodium phosphate was added to the diet of norethandrolone-treated animals, the caries incidence was decreased. The incidence approximated that of control animals fed the caries-test diet supplemented with 2% disodium phosphate. Also, the addition of 2% disodium phosphate to the diet of norethandrolone-treated animals considerably reduced the caries incidence in molar teeth when compared to norethandrolone-treated animals fed the basic caries-test diet only. Thus, it would appear that the addition of 2% disodium phosphate to the diet caused a reversal of norethandrolone cariogenesis under the experimental conditions employed.

According to Solomons, Irving, and Neuman (11), and others, the organic matrix of dentin consists mainly of collagen, with small amounts of mucopolysaccharides similar to chondroitin sulfate. They found that freshly decalcified human dentin could induce formation of hydroxyapatite from calcium-phosphorus solutions. Also, Takuma (12) observed the regular deposition of crystals on the matrix fibrils in human dentin. Therefore, the fibrous protein of dentin and enamel plays a significant role in the deposition and growth of apatite crystals.

Norethandrolone has been shown to exert an effect on the synthesis of chondroiten sulfate and collagenous tissues (13). In addition, upon clinical testing, norethandrolone has been found to increase phosphorus retention (14–16). Since norethandrolone has an effect on phosphorus retention and collagen synthesis, a study of the influence of norethandrolone on phosphorus deposition in rat molar swas undertaken. In an investigation of this type radiological techniques utilizing radio-phosphorus seemed to be the logical procedure.

EXPERIMENTAL

A total of 192 weanling Sprague-Dawley strain albino rats, weighing from 45–50 Gm. each, was divided randomly into four groups of 48 animals each (24 females and 24 males). Sexes were separated and the animals housed three to a cage on screenwire bottoms. All animals were given concentrated vitamin A, D, and E supplement by mouth weekly and distilled water *ad libitum*. All rats were

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TABLE I.—STATISTICAL RELATIONS OF RADIO-PHOSPHORUS CONTENT IN MOLAR TEETH, $P = 0.05$

Time Interval	Male Relations	Female Relations	Male Relations	Female Relations
	Paired Lower First Molar		Paired Upper First Molar	
2	NP[a] = P[b]	NP = P	NP = P	NP = P
2	N[c] = C[d]	N = C	N = C	N = C
4	NP > P	NP = P	NP > P	NP = P
4	N = C	N = C	N = C	N = C
6	NP = P	NP = P	NP = P	NP = P
6	N = C	N = C	N = C	N = C
8	NP = P	NP = P	NP = P	NP = P
8	N = C	N = C	N = C	N = C
	Paired Lower Second Molar		Paired Upper Second Molar	
2	NP = P	NP = P	NP = P	NP = P
2	N = C	N = C	N = C	N = C
4	NP = P	NP = P	NP > P	NP = P
4	N = C	N = C	N = C	N = C
6	NP = P	NP = P	NP = P	NP = P
6	N = C	N = C	N = C	N = C
8	NP = P	NP = P	NP = P	NP = P
8	N = C	N = C	N = C	N = C
	Paired Lower Third Molar		Paired Upper Third Molar	
2	NP = P	NP = P	NP = P	NP = P
2	N = C	N = C	N < C	N = C
4	NP = P	NP = P	NP = P	NP = P
4	N = C	N = C	N = C	N = C
6	NP = P	NP = P	NP = P	NP > P
6	N = C	N = C	N = C	N = C
8	NP = P	NP = P	NP = P	NP = P
8	N = C	N = C	N = C	N = C

[a] NP (Group 1) = norethandrolone plus 2% Na_2HPO_4 added to diet. [b] P (Group 2) = 2% Na_2HPO_4 added to diet. [c] N (Group 3) = norethandrolone added to diet. [d] C (Group 4) = basic diet.

weighed twice weekly. Animal cages were washed with soap and water, rinsed and dried three times a week, while the special feeding cups were washed each morning. All animals were injected intraperitoneally once a week with radio-phosphorus, with the dosage based on 2 μc. per 100 Gm. of body weight. The solutions for injection were prepared from a stock solution of $H_3P^{32}O_4$ in weak HCl by diluting, volumetrically, measured aliquots with 0.001 *M* sodium phosphate to desired volumes. Radio-phosphorus concentrations were prepared not to exceed a volume of 1 ml. per injection per animal.

Animals were given identically weighed quantities of diet with each group fed variations of the basic caries-test diet (Stephan Diet 580)[1] as follows: Group 1 was fed the basic diet to which was added 2% Na_2HPO_4 and norethandrolone, Group 2 was fed the basic diet with added 2% Na_2HPO_4, Group 3 was fed the basic diet with added norethandrolone, and Group 4 was fed the basic diet only.

When 2% Na_2HPO_4 was added to the diet, the salt replaced an equal amount of cane sugar. An analysis of the caries-test diet was obtained to determine the content of phosphorus in the basic diet compared to that in the basic diet plus added Na_2HPO_4. The basic diet contained 0.36% phosphorus, or about half the phosphorus found in the basic diet supplemented with Na_2HPO_4 (0.70% phosphorus). Thus, the animals subsisting on basic diet received considerably less phosphorus than those animals fed the basic diet with added Na_2HPO_4.

Those animals being treated with norethandrolone received a dose closely approximating 5 mg./Kg. of body weight per day during the entire 56-day experimental period. This was accomplished by thoroughly incorporating the drug into the basic caries-test diet. The amount of drug incorporated was based on the average daily weight of the animals in each treated group. A weighed quantity of this diet was placed in each special feeder for those animals receiving the drug sufficient for daily requirements. The following morning refused food was discarded after weighing. Dosage calculations were based on food consumed per day by each cage of three animals over the 56-day period.

At the end of 2 weeks, 48 animals (representing six animals of each sex from each experimental group) were sacrificed with chloroform. Bilateral extractions of the maxillary and mandibular molars were performed. The upper jaw (left and right), first molar teeth were paired and weighed with a model H-5 gram-atic mettler balance. The teeth were dissolved in approximately 1 ml. of nitric acid, with the aid of infrared heat lamps, in a pyrex glass planchet 1 in. in diameter and $^5/_{16}$ in. in depth. This procedure was followed for upper jaw second and third molars, as well as for all lower jaw molars. The samples were dried with infrared heat lamps, the radioactivity determined and corrected for decay.

Determination of radioactivity was accomplished with Tracerlab "1000" scalers connected to shielded end-window geiger tubes. Each sample was counted five times, with sufficient time allowed to accumulate at least 500 counts per determination, thus incurring a probable error of approximately 3%. Additional groups of 48 animals, distributed as in the 2-week study, were sacrificed at 4, 6, and 8-week intervals. The animals were treated in the

[1] Stephan Diet 580: skim milk powder, 32%; cane sugar, 66%; whole dried liver substance N.F., 2%.

TABLE II.—RADIO-PHOSPHORUS LOCATED IN THE MOLAR TEETH OF MALE RATS

Animals	Group	Av. c.p.m./mg. ± S.D. Lower First	Lower Second	Lower Third	Upper First	Upper Second	Upper Third
		2-Wk. Interval					
6	1[a]	15.0 ±1.74	15.1 ±1.09	43.3 ±4.96	13.1 ±1.92	14.3 ±1.86	40.7 ±3.08
6	2[b]	12.8 ±2.85	13.5 ±3.66	40.2 ±9.53	12.6 ±3.93	13.2 ±3.74	39.9 ±10.1
6	3[c]	20.5 ±5.29	21.2 ±0.95	59.6 ±7.58	20.2 ±2.86	19.2 ±3.50	55.0 ±10.7
6	4[d]	22.8 ±1.11	20.9 ±4.70	62.7 ±4.45	21.5 ±4.87	21.1 ±5.71	67.9 ±6.83
		4-Wk. Interval					
5	1	16.1 ±1.66	16.8 ±3.85	30.7 ±0.81	15.9 ±0.83	15.1 ±1.49	31.0 ±0.69
6	2	13.6 ±1.34	13.1 ±1.44	27.5 ±2.42	13.1 ±1.14	12.6 ±1.64	27.6 ±3.71
4	3	21.8 ±3.06	21.2 ±1.28	41.8 ±4.67	20.9 ±2.65	17.7 ±0.83	41.0 ±4.94
5	4	22.7 ±1.23	20.7 ±1.97	43.1 ±4.27	21.0 ±2.28	20.4 ±2.55	42.8 ±5.06
		6-Wk. Interval					
5	1	17.1 ±2.03	15.8 ±2.18	24.3 ±2.00	15.9 ±1.97	15.8 ±2.13	23.8 ±2.50
5	2	15.1 ±0.86	14.8 ±1.35	24.5 ±1.17	15.0 ±0.74	14.4 ±1.96	23.5 ±1.88
6	3	25.7 ±3.87	24.0 ±3.04	34.7 ±6.81	22.3 ±3.01	20.8 ±2.86	33.9 ±5.99
6	4	22.3 ±4.41	25.2 ±5.26	35.8 ±3.39	24.1 ±2.13	22.6 ±0.97	34.8 ±3.49
		8-Wk. Interval					
6	1	13.2 ±1.24	13.1 ±1.09	17.1 ±0.57	13.0 ±1.70	12.2 ±1.05	17.4 ±0.78
6	2	13.9 ±1.69	14.3 ±3.29	17.4 ±1.44	13.1 ±0.95	11.9 ±0.99	16.6 ±0.80
6	3	23.9 ±1.73	22.6 ±2.11	29.4 ±2.26	21.7 ±2.58	20.4 ±1.46	24.4 ±5.62
6	4	21.8 ±2.87	21.0 ±2.85	27.4 ±2.83	20.8 ±3.15	19.9 ±2.96	24.2 ±6.80

[a] Group 1 = norethandrolone plus 2% Na_2HPO_4 added to diet. [b] Group 2 = 2% Na_2HPO_4 added to diet. [c] Group 3 = norethandrolone added to diet. [d] Group 4 = basic diet.

same manner as those rats killed after 2 weeks of experimentation.

One male animal died in each of the following groups: Group 1 and 4 of the 4-week time interval, and Group 1 and 2 of the 6-week time interval. Two male animals died in the Group 3 of the 4-week study.

RESULTS AND DISCUSSION

Table I shows the relation of the radio-phosphorus content in the various male and female molar teeth based on statistical comparisons. Tables II and III show the radio-phosphorus located in the molar teeth of male and female rats, respectively.

It would seem that, under the conditions of this experiment, norethandrolone had no effect on radio-phosphorus deposition in rat molars in animals on high or low phosphorus caries-test diets compared with their respective controls. Table I shows that the radio-phosphorus levels in the norethandrolone-treated animals fed a high phosphorus diet (NP) equalled the radio-phosphorus levels in those animals on a high phosphorus diet only (P), in 44 out of 48 comparisons. In 47 out of 48 comparisons no difference existed that was statistically significant between the radio-phosphorus content of the teeth of animals treated with norethandrolone (N) and the respective controls (C) at 2, 4, 6, and 8-week intervals.

Radioassay at 2-week intervals was conducted in an attempt to determine if differences existed in the effect of norethandrolone in the more recently erupted teeth compared with those previously formed. The relations of phosphorus uptake in the norethandrolone-treated animals, with or without added phosphorus, to controls were similar at all time intervals. Therefore, a change in the effect of norethandrolone on radio-phosphorus uptake in rat molars compared with controls over a period of time could not be shown. Also, a difference in the effect of the drug on radio-phosphorus uptake between first, second, and third molars compared with controls could not be shown.

It has been stated that norethandrolone had a phosphorus retention effect when administered clinically (14–16). If this is true in the rat, under the experimental conditions in this study, then apparently the increased phosphorus retention had little or no effect upon the uptake of radio-phosphorus in the rat molar. This can be seen from the fact that radiation levels in the norethandrolone animals were equivalent to those in the control group both with and without high phosphorus diets. A further study is in progress to determine if norethandrolone has a phosphorus retention effect in the rat under our experimental conditions.

The most widely accepted opinion of the crystal structure and chemical composition of tooth mineral

is that it is basically hydroxyapatite, *i.e.*, $Ca_{10}(PO_4)_6(OH)_2$, although certain deviations, such as substitution of carbonate for phosphate and fluoride for hydroxide, are recognized.

Since phosphorus is an integral part of the hydroxyapatite crystal, it may be assumed that the amount of P^{32} present in molar teeth of rats upon administration of radio-phosphorus is dependent upon three factors. These are (*a*) the production of crystalline hydroxyapatite and its deposition in the matrix, (*b*) ion-transfer across the crystal-solution interface along with intracrystalline exchange, and (*c*) resorption (17).

Little has been done to study the effect of drugs on the physical processes of ion-transfer and intracrystalline exchange in osseous material. It will be necessary at this point, therefore, to assume similarity in this process among all experimental groups within the limits of biological error. There is no reason to assume similarity among experimental groups as far as the metabolic processes are concerned and, therefore, we are relating P^{32} uptake as being indicative of calcification.

Table I shows that the radio-phosphorus content in the norethandrolone-treated rats equalled that found in the respective control groups. This was observed in comparisons of molar teeth from rats fed basic diet plus norethandrolone and Na_2HPO_4 (NP) to rats fed basic diet and Na_2HPO_4 (P), and comparisons of molars from animals fed basic diet and norethandrolone (N) to animals fed basic diet only (C). It would appear that, under the conditions of this experiment, norethandrolone had little or no effect upon calcification.

If norethandrolone had a positive effect upon the synthesis of the collagenous fibrils making up the protein matrix and the mucopolysaccharides making up ground substance, then it could be assumed that a greater rate of calcification would take place. This would seem to be true since Frank (18) showed that hydroxyapatite crystals are laid down on newly formed fibrils of the organic matrix. Also, Belanger (19) found that hydroxyapatite crystal growth occurs soon after the formation of fibrous protein. Carstrom (20) has stated that collagen controls calcification by effecting crystallization of apatite in dentin. In addition, Sobel and his collaborators (21) suggest that, while collagen induces nucleation, the complete system is more complex and probably includes sulfated mucopolysaccharide or mucoprotein. Although it cannot be conclusively stated from this one experiment, it would appear that norethandrolone had little or no effect on collagen synthesis in the rat molar. Further study using radio-sulfur and normal diets and analysis of the radio-sulfur uptake might throw additional light on this particular aspect of the problem. In summary of the above, the results of

TABLE III.—RADIO-PHOSPHORUS LOCATED IN THE MOLAR TEETH OF FEMALE RATS

Animals	Group	Av. c.p.m./mg. ± S.D. Lower First	Lower Second	Lower Third	Upper First	Upper Second	Upper Third
		2-Wk. Interval					
6	1[a]	12.9 ±1.51	12.9 ±1.58	38.4 ±7.00	13.2 ±1.41	12.4 ±1.35	37.3 ±6.99
6	2[b]	13.6 ±1.88	12.9 ±2.08	36.4 ±4.55	14.1 ±1.39	12.7 ±1.81	36.0 ±5.56
6	3[c]	21.5 ±2.68	21.0 ±3.54	65.4 ±7.29	22.4 ±1.12	21.1 ±2.65	57.4 ±9.15
6	4[d]	20.8 ±2.96	20.3 ±2.45	62.3 ±5.07	22.0 ±1.36	20.3 ±2.06	57.5 ±6.71
		4-Wk. Interval					
6	1	13.5 ±1.71	12.6 ±1.93	26.7 ±3.87	12.9 ±1.37	12.2 ±2.02	25.4 ±3.98
6	2	13.7 ±1.58	13.1 ±1.03	23.9 ±1.33	13.5 ±1.48	11.8 ±1.59	24.6 ±1.94
6	3	22.7 ±1.86	22.4 ±2.58	40.8 ±6.89	20.9 ±2.35	20.0 ±1.74	37.4 ±7.69
6	4	21.7 ±2.26	21.1 ±2.40	40.6 ±2.98	20.4 ±1.44	19.2 ±2.21	38.7 ±3.85
		6-Wk. Interval					
6	1	16.0 ±1.15	14.5 ±1.39	24.8 ±1.11	15.8 ±1.50	14.0 ±1.10	24.6 ±2.91
6	2	15.6 ±1.80	14.7 ±1.01	22.6 ±2.24	14.5 ±0.50	13.5 ±1.05	21.3 ±1.45
6	3	24.5 ±1.81	23.3 ±2.21	37.2 ±3.55	25.0 ±2.45	21.5 ±2.16	36.5 ±2.83
6	4	24.8 ±1.67	22.0 ±1.41	35.7 ±3.32	23.1 ±2.21	22.1 ±1.08	34.3 ±4.05
		8-Wk. Interval					
6	1	13.2 ±0.92	11.8 ±1.08	17.0 ±0.53	13.1 ±0.85	12.8 ±1.07	16.8 ±1.40
6	2	12.3 ±1.43	11.8 ±1.63	16.1 ±1.27	13.4 ±2.51	12.0 ±1.46	15.8 ±1.40
6	3	22.8 ±2.90	18.7 ±0.72	28.6 ±4.65	21.5 ±2.50	21.2 ±2.82	28.0 ±2.54
6	4	22.9 ±1.45	21.7 ±3.37	28.1 ±2.45	21.2 ±2.06	21.3 ±1.68	28.5 ±1.77

[a] Group 1 = norethandrolone plus 2% Na_2HPO_4 added to diet. [b] Group 2 = 2% Na_2HPO_4 added to diet. [c] Group 3 = norethandrolone added to diet. [d] Group 4 = basic diet.

this experiment would indicate that the caries resistance of norethandrolone-treated animals fed diets containing 2% disodium phosphate is not related to phosphorus deposition, rate of calcification, or collagen synthesis in rat molars.

A study of Tables II and III shows that the radio-phosphorus content of the teeth in the animals on high phosphorus diets was at a lower value than the radio-phosphorus content in the teeth of animals on low phosphorus diets. This effect was not related to the presence or absence of norethandrolone. This was true in the majority of all comparisons made.

There probably is no one explanation for these results. One reason could be a dilution factor occurring in those animals on a high phosphorus diet. Since the use of a particular phosphate ion from the metabolic pool in synthesis is a random procedure, the greater ratio of nonradioactive ions to radioactive ions would tend to lower the activity in the synthesized dental tissue in those animals on a high phosphorus diet.

Probably of greater importance is the relation of added dietary phosphorus to urinary phosphate elimination. Sivachenko (22) found that heavy administration of nonradioactive phosphorus and calcium salts was most effective in increasing the rate of P^{32} elimination in rats.

Goldman and Bassett (23) studied renal regulation of phosphorus excretion in male patients with normal phosphorus metabolism. They found that daily oral administration of a mixture of sodium and potassium phosphates caused a significant increase in the serum phosphorus level in the patients. This was followed by an increase in urinary phosphorus.

It would appear that the difference in activity in the teeth of high-phosphorus *versus* low-phosphorus animals might be accounted for by these above factors.

CONCLUSIONS

The influence of norethandrolone on radio-phosphorus deposition in rat molars of animals on high and low phosphorus caries-test diets was determined.

Radio-phosphorus levels in the molar teeth of norethandrolone-treated animals fed a high phosphorus diet equalled the radio-phosphorus levels in those animals on a high phosphorus diet alone.

No statistically significant difference existed between the radio-phosphorus content in the molar teeth of animals treated with norethandrolone and the respective control animals.

The radio-phosphorus content of the molar teeth in animals on high phosphorus diets was at a lower value than the radio-phosphorus content in the teeth of animals fed low phosphorus diets. This effect was not related to the presence or absence of norethandrolone but might be accounted for by ion dilution or an increased rate of phosphorus elimination.

No change in the effect of norethandrolone on radio-phosphorus uptake in rat molars, with or without added phosphorus, over a time interval was shown.

A difference in the effect of norethandrolone on radio-phosphorus uptake between first, second, and third molar teeth compared with respective controls was not observed in either animals fed a high phosphorus diet or rats on a low phosphorus diet.

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Antibacterial Agents Not Presently Employed as Preservatives in Ophthalmic Preparations Found Effective Against *Pseudomonas aeruginosa*

By S. R. KOHN†, LOUIS GERSHENFELD, and MARTIN BARR

Fifty-one chemical substances, not heretofore employed as preservatives in ophthalmic solutions, were examined for their effectiveness as antibacterial agents against 13 different strains of *Pseudomonas aeruginosa*. *In vitro* and *in vivo* methods were employed. Eleven of these agents showed a satisfactory sterilizing time against these strains. Further study of the following is indicated to determine their practical use as effective preservatives in ophthalmic preparations: Intexsan LB, Intexsan MB, Dichloran, DMBC, Cetol, Arquad 16, Virac, Betadine, Biopal VRO, chlorhexidine, and colistin.

A COMMONLY FOUND and hazardous contaminant in ophthalmic solutions is *Ps. aeruginosa*. The latter causes a serious type of corneal ulcer encountered in ophthamology, often resulting in the loss of the infected eye.

The chemicals which are commonly employed as antibacterial agents in multidose ophthalmic solutions were investigated for their effectiveness against 13 strains of *Ps. aeruginosa*, utilizing in each instance inactivating media of determined neutralizing activity for subculturing (1). Only benzalkonium chloride had a sterilizing time of less than 1 hour against the 13 strains of bacteria (1). An antibacterial substance which has a sterilizing time greater than 1 hour may be arbitrarily considered to be too slow-acting for use as a preservative in multidose ophthalmic solutions. Accordingly, this investigation was undertaken to determine the length of time required for sterilization by various antibacterial agents not heretofore employed as preservatives in ophthalmic solutions against *Ps. aeruginosa*. The main purpose was to ascertain whether any of these chemicals were capable of sterilizing in less than 1 hour.

EXPERIMENTAL[1]

Antibacterial Agents.—The chemicals studied for their effectiveness as antibacterial agents for ophthalmic solutions against *Ps. aeruginosa* were: 37 quaternary ammonium compounds: myristyl gamma-picolinium chloride,[2] Hyamine 1622,[3] Hyamine 10-X,[4] Hyamine 3500,[5] Hyamine 2389,[6] Emcol E-11,[7] Emcol E-607,[8] Emcol E-6075,[9] Ruson,[10] Tetrosan 3, 4D,[11] Tetrosan 60%,[12] Onyxide 75%,[13] BTC-776,[14] Intexsan MB-50,[15] Intexsan LB-50,[16] Intexsan LCB-50,[17] Intexsan LQ-75,[18] Intexsan OE-75,[19] Dichloran,[20] Cetol,[21] Bromat,[22] Bretol,[23] Germitol,[24] SD-75,[25] CTA-Stearate,[26] cetyl pyridinium bromide,[27] Laurol,[28] DMBC,[29] Arquad 12,[30] Arquad 16,[31] Arquad 18,[32] Arquad C,[33] Arquad S,[34] Arquad T,[35] Arquad HT,[36] cetyl pyridinium chloride,[37] and lauryl pyridinium chloride;[38] eight amphoteric surface-active agents: Tego 103-S,[39] Janusol,[40] Miranol OM-SF Concentrate,[41] and Miranol C2M Concentrate,[42] Miranol MSA Modified,[43] Miranol 2MCA Modified,[44] Product BCO,[45] and Product BDO;[46] three iodophors: Biopal VRO,[47] Virac,[48] and Betadine;[49] and a group of miscellaneous agents including Agosan,[50] chlorhexidine,[51] and colistin.[52]

Test Bacteria.—Two dilutions (1:10 and 1:1000) of 24-hour-old cultures of 13 different strains of *Ps. aeruginosa* (1) were used in this study. The bacteria were identified and the cultures prepared as previously described (1).

In Vitro Method.—The chemicals were studied for their effectiveness as bactericidal agents against 13 strains of *Ps. aeruginosa* using a procedure as noted in *Experiment III*, described in a previous study (1). The inactivating media employed for the purpose of neutralizing the antibacterial agents were evaluated using experiments which were also previously described (1). The most effective neutralizing media for use in these studies are listed in Table I.

In Vivo Method.—The purpose and the method employed for verifying the *in vitro* results (sterilizing times) for each of the antibacterial agents using an *in vivo* procedure have been reported (1). This same technique was used in this study.

RESULTS

Table II presents a summary of the results obtained. All of the antibacterial agents listed in this table gave results which indicate that they have a sterilizing time against 13 different strains of *Ps. aeruginosa* equal to or better than 1:5000 benzalkonium chloride. The sterilizing times (1) listed have been obtained under the severe *in vitro* test conditions and have been verified by an *in vivo* technique. In the latter procedure, those solutions of chemicals which produced growth in the subculture media produced ocular infections; those which did not produce growth did not produce ocular infections. The agents which gave longer sterilizing times than that given by 1:5000 benzalkonium chloride (45 minutes) are not listed in Table II.

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[1] See *Appendix* for footnotes to compounds listed.

TABLE I.—NEUTRALIZING MEDIA MOST EFFECTIVE

For "Quats"—Medium V (pH 6.7) (1)	
Lecithin[a]	0.5 Gm.
Tween 80[b]	3 Gm.
Nutrient broth, dehydrated[c]	0.8 Gm.
Purified water *q.s. ad.*	100 ml.
For Amphoteric Surfactants—Medium I (pH 6.8) (1)	
Nutrient broth, dehydrated[c]	0.8 Gm.
Purified water *q.s. ad.*	100 ml.
For Iodophors—Medium XIX (pH 6.7) (1)	
Sodium thiosulfate[d]	0.5 Gm.
Lecithin[a]	0.5 Gm.
Tween 80[b]	3 Gm.
Medium I *q.s. ad.*	100 ml.
For Agosan—Medium VIII (pH 7.1) (1)	
Fluid thioglycollate medium, dehydrated[c]	2.93 Gm.
Purified water *q.s. ad.*	100 ml.
For Chlorhexidine—Medium XX (pH 6.8) (1)	
Sodium lauryl sulfate[e]	0.5 Gm.
Medium I *q.s. ad.*	100 ml.
For Colistin—Medium III (pH 6.8) (1)	
Lecithin	0.5 Gm.
Glycerin	4 Gm.
Medium I *q.s. ad.*	100 ml.

[a] Lecithin, (Ex Ovo Soluble) Pfansteihl Laboratories, Inc., Waukegan, Ill. [b] Polyoxyethylene (20) sorbitan mono-oleate, Atlas Powder Co., Wilmington, Del. [c] Difco Laboratories, Inc., Detroit, Mich. [d] Sodium thiosulfate, reagent, Merck and Co., Rahway, N. J. [e] Marketed as Duponol C, by E. I. Du Pont de Nemours & Co., Inc., Wilmington, Del.

DISCUSSION

Among the 37 quaternary ammonium compounds studied, the following quats were found to be equal to or better than benzalkonium chloride in their bactericidal effectiveness against *Ps. aeruginosa:* Intexsan LB, Intexsan MB, DMBC, Cetol, Dichloran, and Arquad 16. Their sterilizing times were 45 minutes or less. However, before these compounds are employed as antibacterial preservatives in ophthalmic solutions, their toxicity, ocular irritability, stability, and compatibilities with the common ophthalmic drugs and vehicles must be determined. It should be mentioned that Medium V inactivated 1:1000 dilutions of the "quats," the strongest concentration studied.

The amphoteric surfactants possess either a positive or negative charge in solution depending on the pH of the latter. Based on the probability that an acid solution would cause the amphoteric surfactants to assume a positive charge, they were studied both in aqueous solutions and in aqueous solutions containing 1% boric acid. However, only one of these compounds, namely Tego 103-S, when used in a concentration of 1:1000, possessed antibacterial activity against *Ps. aeruginosa* after 24 hours of contact in either aqueous solutions or aqueous solutions containing 1% boric acid. Furthermore, the sterilizing time against *Ps. aeruginosa* was too long (9 hours), revealing no improvement over benzalkonium chloride.

Inasmuch as Virac is a quaternary-iodine complex, the inactivating medium employed in the investigation of the iodophors was the same medium as that employed for the quaternary ammonium compounds but with the addition of sodium thiosulfate to inactivate the iodine. The neutralizing medium, Medium XIX, was capable of neutralizing iodophors in concentrations of 500 p.p.m. of iodine, the strongest concentration tested.

It was noted that the iodophors, in either aqueous solutions or in aqueous solutions containing 1% boric acid, were effective against *Ps. aeruginosa* in concentrations as low as 25 p.p.m. of available iodine. It is known that iodophors are more stable in acid solutions and hence more effective. It should be emphasized that aqueous solutions containing 25 p.p.m. of available iodine had pH values in the acid range. Thus, there was no difference in the antibacterial effectiveness of the iodophors against *Ps. aeruginosa* in aqueous solutions or in aqueous solutions containing 1% boric acid. However, it was observed that the iodophors in aqueous solutions containing 10 p.p.m. of available iodine did not have pH values in the acidic range. Thus, the sterilizing times for the iodophors against *Ps. aeruginosa* in aqueous solutions (10 p.p.m. available iodine) required more than 1 hour of contact, while the same iodophors in aqueous solutions containing 1% boric acid were effective against *Ps. aeruginosa* after 30 minutes of contact.

From the findings in this study, it would appear that Virac, Betadine, and Biopal VRO may possibly be used as effective preservatives in ophthalmic solutions. However, as stated previously for the quaternary ammonium compounds, other properties of these iodophors, which are desired for antibacterial preservatives used in ophthalmic solutions, must be thoroughly investigated before employing the iodophors in such solutions. Medium VIII, fluid thioglycollate medium, was found to be an

TABLE II.—STERILIZING TIMES AGAINST *Ps. aeruginosa* (10^8) FOR CHEMICAL AGENTS NOT HERETOFORE EMPLOYED AS PRESERVATIVES IN OPHTHALMIC SOLUTIONS WHICH SHOW PROMISE FOR FURTHER STUDY

Chemical Agent	Concn.	Sterilizing Time, Min.
Intexsan LB	1:5000	45
Intexsan MB	1:5000	30
Dichloran	1:5000	45
DMBC	1:5000	45
Cetol	1:5000	30
Arquad 16	1:5000	45
Virac[a]	100 p.p.m. I_2	15
Virac[a]	50 p.p.m. I_2	15
Virac[a]	25 p.p.m. I_2	15
Betadine[a]	100 p.p.m. I_2	15
Betadine[a]	50 p.p.m. I_2	15
Betadine[a]	25 p.p.m. I_2	15
Biopal VRO[a]	100 p.p.m. I_2	15
Biopal VRO[a]	50 p.p.m. I_2	15
Biopal VRO[a]	25 p.p.m. I_2	15
Virac[b]	10 p.p.m. I_2	30
Betadine[b]	10 p.p.m. I_2	30
Biopal VRO[b]	10 p.p.m. I_2	30
Chlorhexidine	1:50,000	30
Chlorhexidine	1:25,000	15
Chlorhexidine	1:10,000	15
Chlorhexidine	1:5000	15
Colistin	1000 units/ml.	15
Colistin	500 units/ml.	15
Colistin	250 units/ml.	45

[a] In aqueous solution and in 1% boric acid solution. [b] In 1% boric acid solution only.

effective inactivating agent for Agosan, neutralizing 1% w/v of the substance, the highest concentration studied. This was to be expected, since the sulfhydryl groups in the thioglycollate are reactive not only with mercurial ions but also with other metallic ions.

Agosan, in a concentration of 0.5% possessed a sterilizing time against *Ps. aeruginosa* of 6 hours, a time period which is not an improvement over that for 1:5000 benzalkonium chloride.

Medium XX, which contained 0.5% sodium lauryl sulfate in nutrient broth medium, neutralized chlorhexidine in concentrations up to 1:1000, the strongest concentration studied.

Chlorhexidine in concentrations of 1:50,000 had a sterilizing time of 30 minutes for *Ps. aeruginosa*. Stronger concentrations had a faster sterilizing time. Further investigations are indicated for chlorhexidine as they concern toxicity, irritability, stability, and compatibility with the common medicaments and vehicles.

Medium III was found to be a good inactivating agent for colistin, neutralizing 2000 units/ml., the strongest concentration studied. Medium III was selected because colistin, like polymyxin B sulfate, is a polypeptide and carries a positive charge.

Colistin, in a concentration of 250 units/ml., had a sterilizing time of 45 minutes against *Ps. aeruginosa*. In stronger concentrations, the sterilizing time was reduced considerably.

SUMMARY

Fifty-one chemical substances, not heretofore employed as preservatives in ophthalmic solutions, were studied for their effectiveness against 13 different strains of *Ps. aeruginosa*. *In vitro* and *in vivo* methods were employed. Sterilizing times were determined in each instance and these were compared with that of benzalkonium chloride.

Thirty-seven quaternary ammonium compounds were investigated for their antibacterial effectiveness against *Ps. aeruginosa*. Of these, only six were found to possess equal or superior activity against *Ps. aeruginosa* compared to benzalkonium chloride. These included Intexsan LB, Intexsan MB, Dichloran, DMBC, Cetol, and Arquad 16.

Eight amphoteric surfactants were studied for their antibacterial effectiveness against *Ps. aeruginosa*. They were all found to be inadequate for use as antibacterial preservatives in ophthalmic solutions when compared to benzalkonium chloride.

Three iodophors, Virac, Betadine, and Biopal VRO were investigated and found to possess sterilizing times of less than 1 hour against *Ps. aeruginosa*.

Three other antibacterial agents, namely Agosan, chlorhexidine, and colistin, were investigated for their antibacterial activity against *Ps. aeruginosa*. Chlorhexidine and colistin were each found to possess sterilizing times of less than 1 hour against *Ps. aeruginosa*. Agosan required a longer sterilizing time.

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APPENDIX

[2] Marketed as Quatresin by The Upjohn Co., Kalamazoo, Mich.

[3] Di-isobutyl phenoxy ethoxy ethyl dimethyl benzyl ammonium chloride, Rohm and Haas Co., Philadelphia, Pa.

[4] Di-isobutyl cresoxy ethoxy ethyl dimethyl benzyl ammonium chloride, Rohm and Haas Co., Philadelphia, Pa.

[5] A 50% w/w hydroalcoholic solution of alkyl (C_{14}, 50%; C_{12}, 40%; C_{16}, 10%) dimethyl benzyl ammonium chloride, Rohm and Haas Co., Philadelphia, Pa.

[6] Fifty per cent w/w aqueous solution containing 40% methyl dodecyl benzyl trimethyl ammonium chloride and 10% methyl dodecyl xylene bis(trimethyl ammonium chloride), Rohm and Haas Co., Philadelphia, Pa.

[7] Fifty per cent w/w aqueous solution containing alkyl (C_8 to C_{18}, predominately C_{12}) benzyl trimethyl ammonium chloride, Witco Chemical Co., Inc., Chicago, Ill.

[8] N (lauroyl colaminoformyl methyl)pyridinium chloride Witco Chemical Co., Inc., Chicago, Ill.

[9] N(stearoyl colaminoformyl methyl)pyridinium chloride, Witco Chemical Co., Inc., Chicago, Ill.

[10] Aqueous solution containing 3.2% w/v of N(methyl heptyl colaminoformyl methyl)pyridinium chloride, Ruson Laboratories, Inc., Portland, Ore.

[11] Alkyl, dimethyl 3,4 dichloro-benzyl ammonium chloride, 60% w/w liquids, Onyx Chemical Corp., Jersey City, N. J.

[12] A 60% w/w liquid containing a mixture of alkyl dimethyl 3,4 dichlorobenzyl ammonium bromide and alkenyl dimethyl ethyl ammonium bromide, Onyx Chemical Corp., Jersey City, N. J.

[13] Alkenyl (predominately C_{18}) dimethyl ethyl ammonium bromide, a 75% w/w paste, Onyx Chemical Corp., Jersey City, N. J.

[14] Fifty per cent w/w liquid containing a mixture of alkyl dimethyl benzyl ammonium chlorides, Onyx Chemical Corp., Jersey City, N. J.

[15] Fifty per cent w/w liquid containing alkyl (predominately C_{14}, C_{16}, C_{12}) dimethyl benzyl ammonium chlorides, Intex Chemical Corp., Lodi, N. J.

[16] Fifty per cent w/w liquid containing alkyl (predominately C_{12}, C_{14}, C_{16}) dimethyl benzyl ammonium chlorides, Intex Chemical Corp., Lodi, N. J.

[17] Fifty per cent w/w liquid containing alkyl (predominately C_{12}, C_{14}, C_{16}) dichlorobenzyl ammonium chlorides, Intex Chemical Corp., Lodi, N. J.

[18] Seventy-five per cent w/w liquid containing alkyl (predominately C_{12}, C_{14}, C_{16}) isoquinolinium bromide, Intex Chemical Corp., Lodi, N. J.

[19] Seventy-five per cent w/w paste containing alkyl (predominately C_{13}) dimethyl ethyl ammonium bromides, Intex Chemical Corp., Lodi, N. J.

[20] Fifty per cent w/w liquid containing alkyl (C_8 to C_{18}) dimethyl dichlorobenzyl ammonium chloride, Fine Organics, Inc., Lodi, N. J.

[21] Cetyl dimethyl benzyl ammonium chloride, Fine Organics, Inc., Lodi, N. J.

[22] Cetyl trimethyl ammonium bromide, Fine Organics, Inc., Lodi, N. J.

[23] Cetyl dimethyl ethyl ammonium bromide, Fine Organics, Inc., Lodi, N. J.

[24] Sixty per cent w/w liquid containing lauryl dimethyl benzyl ammonium chloride, Fine Organics, Inc., Lodi, N. J.

[25] Seventy-five per cent w/w liquid containing alkenyl (predominately C_{18}) dimethyl ethyl ammonium bromides, Fine Organics, Inc., Lodi, N. J.

[26] Cetyl trimethyl ammonium stearate, Fine Organics, Inc., Lodi, N. J.

[27] Marketed by Fine Organics, Inc., Lodi, N. J.

[28] Lauryl dimethyl benzyl ammonium bromide, Fine Organics, Inc., Lodi, N. J.

[29] Fifty per cent w/w liquid containing lauryl dimethyl benzyl dimethyl ammonium chloride, Fine Organics, Inc., Lodi, N. J.

[30] Fifty per cent w/w liquid containing alkyl (C_{12}, 90%) trimethyl ammonium chloride, Armour Industrial Chemical Co., Chicago, Ill.

[31] Fifty per cent w/w liquid containing alkyl (C_{16}, 90%) trimethyl ammonium chloride, Armour Industrial Chemical Co., Chicago, Ill.

[32] Fifty per cent w/w liquid containing alkyl (C_{18}, 93%) trimethyl ammonium chloride, Armour Industrial Chemical Co., Chicago, Ill.

[33] Fifty per cent w/w liquid containing alkyl (C_8 to C_{18} predominately C_{12}) trimethyl ammonium chloride, Armour Industrial Chemical Co., Chicago, Ill.

[34] Fifty per cent w/w liquid containing alkyl (C_{16} to C_{18}, predominately unsaturated C_{18}) trimethyl ammonium chloride, Armour Industrial Chemical Co., Chicago, Ill.

[35] Fifty per cent w/w liquid containing alkyl (C_{14}, C_{16}, and C_{18}, predominately unsaturated C_{18}) trimethyl ammonium chlorides, Armour Industrial Chemical Co., Chicago, Ill.

[36] Fifty per cent w/w liquid containing alkyl (C_{14}, C_{16}, C_{18}, predominately saturated C_{18}) trimethyl ammonium chloride, Armour Industrial Chemical Co., Chicago, Ill.

[37] Marketed by Fine Organics, Inc., Lodi, N. J.

[38] Marketed by Fine Organics, Inc., Lodi, N. J.

[39] Salt of octyl amino ethyl glycine, Th. Goldschmidt AG, Essen, Germany.

[40] Mixture of lauryl and myristyl esters containing both primary amine and sulfate groups, Synthetic Chemicals, Inc., Paterson, N. J.

[41] Thirty-five per cent w/w liquid containing sodium, 1-oleyl 2-hydroxy 2 hydroxymethyl ethylene cycloimidinium 2-methylene carboxylate, Miranol Chemical Co., Inc., Irvington, N. J.

[42] Forty-nine per cent w/w liquid containing sodium, 1-undecyl 2-hydroxy 2-sodium ethoxymethylene carboxylate ethylene cycloimidinium 2-methylene carboxylate, Miranol Chemical Co., Inc., Irvington, N. J.

[43] Forty-seven per cent w/w liquid containing sodium 1-nonyl 2-lauryl sulfate 2-hydroxyethyl ethylene cycloimidinium 2-methylene carboxylate, Miranol Chemical Co., Inc., Irvington, N. J.

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[47] Iodine-nonyl phenoxypolyoxyethylene ethanol complex, in liquid form and containing 20% available iodine, Antara Chemicals, Division of General Aniline and Film Corp., New York, N. Y.

[48] N(methyl heptyl colamino-formyl methyl)pyridinium chloride containing coupled iodine, in liquid form and containing 0.6% available iodine, Ruson Laboratories, Inc., Portland, Ore.

[49] Polyvinylpyrrolidone-iodine complex, in liquid form and containing 1% available iodine, Tailby-Nason Co., Inc., Dover, Del.

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[51] Diacetate salt of bis (*p*-chlorophenyl diquanido)-hexane. Marketed as Hibitane by Ayerst Laboratories, New York, N. Y.

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THE RELATIVE RATES of second-order alkaline hydrolysis of atropine (1), homatropine (2), atropine methylbromide, and homatropine methylbromide (3) have been reported previously. The purpose of this investigation was to study further the influence of inductive and steric factors on the hydrolytic cleavage of these esters. The kinetics of the hydroxyl ion catalyzed hydrolysis of nor-atropine were studied in order to establish hydrolytic rates for what might be considered the base compound. Tropine phenylacetate, tropine phenoxyacetate, and tropine *p*-nitrobenzoate were prepared and rates of hydrolysis determined. In addition, atropine ethylbromide and atropine benzylchloride were prepared and studied.

THEORY

Previous studies (1–3) have shown hydroxyl ion catalyzed hydrolysis to occur with tropine esters. Since nor-atropine, tropine phenylacetate, tropine phenoxyacetate, and tropine *p*-nitrobenzoate can exist as the free base or as the protonated form in solution, two hydrolytic pathways are possible. For convenience, hydrolysis of the free base will be designated Reaction 1, hydrolysis of the protonated form of the ester will be designated Reaction 2.

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In accord with the theoretical concepts employed by Higuchi, *et al.* (4), in their study on procaine, the reaction kinetics may be

$$-\log t_{1/2} = \log (OH^-) - \log [K_b + (OH^-)] + \log \left[\frac{k_1(OH^-)}{0.693} + \frac{k_2 \cdot K_b}{0.693}\right] \quad \text{(Eq. 1)}$$

where k_1 and k_2 are the second-order rate constants at high pH (Reaction 1) and low pH (Reaction 2), respectively, and K_b is the dissociation constant of the base.

At relatively high hydroxyl ion concentration, where $OH^- >> K_b$, Eq. 1 reduces to

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It is apparent, then, that at high pH and at low pH the rate of hydrolysis should be directly proportional to hydroxyl ion concentration even though the mechanism of hydrolysis changes.

In the case of esters containing quaternary nitrogen, such as atropine ethylbromide and atropine benzylchloride, the mechanism of hydrolysis remains the same throughout the entire pH range.

EXPERIMENTAL

Materials.—Nor-atropine sulfate (K and K Laboratories) was recrystallized from ethanol and water.

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In the case of esters containing quaternary nitrogen, such as atropine ethylbromide and atropine benzylchloride, the mechanism of hydrolysis remains the same throughout the entire pH range.

EXPERIMENTAL

Materials.—Nor-atropine sulfate (K and K Laboratories) was recrystallized from ethanol and water.

TABLE I.—BUFFER SOLUTIONS USED

pH Range	Compn.
6.99–7.97	$M/15$ KH_2PO_4-Na_2HPO_4
8.03–9.89	$M/10$ H_3BO_3 + $M/10$ KCl + NaOH
10.01–10.51	$M/20$ $Na_2B_4O_7$-Na_2CO_3

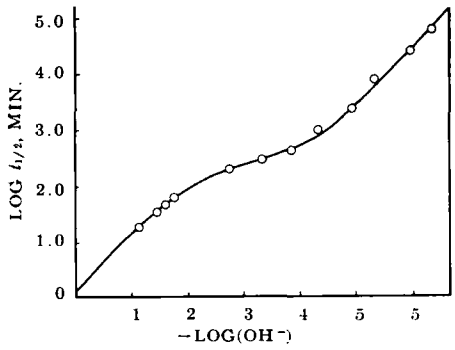

Fig. 1.—Half life of nor-atropine as a function of (OH^-) at 30°C. Solid line, theoretical; points, experimental.

Tropine phenylacetate, tropine phenoxyacetate, and tropine *p*-nitrobenzoate were prepared by reacting tropine hydrochloride with the appropriate acid chloride as described below. Phenylacetyl chloride was prepared according to the procedure of Auwers (5), phenoxyacetyl chloride was prepared according to the procedure of Rosenmund and Zetzsche (6), and *p*-nitrophenylacetyl chloride was obtained commercially.

A 2.0-Gm. quantity of the appropriate acid chloride was added to 2.5 Gm. of tropine hydrochloride in 20 ml. of dry chloroform. The reaction mixture was refluxed gently for 12 hours until evolution of hydrochloric acid ceased. The reaction mixture was extracted several times with water; the aqueous solution of ester salt was then made alkaline with 6% ammonia solution and immediately extracted with chloroform. Chloroform solutions were extracted four times with 5% hydrochloric acid solution, and the aqueous solution evaporated to dryness under vacuum (29°). The residue was dissolved in absolute ethanol, filtered, and anhydrous ether added until the solution just became hazy. Crystals which formed over 36 hours were filtered off and dried at 80°. The hydrochloride salt of the ester was recrystallized twice following the procedure outlined above and dried at 100°. Yields were poor but sufficient ester was obtained for hydrolytic studies.

Tropine phenylacetate hydrochloride, m.p. 197°.

Anal.—Calcd for $C_{16}H_{21}NO_2 \cdot HCl$: C, 64.96; H, 7.49; N, 4.73; Cl, 11.98. Found: C, 64.96; H, 7.50; N, 4.74; Cl, 12.06.

Tropine phenoxyacetate hydrochloride, m.p. 180°.

Anal.—Calcd. for $C_{16}H_{21}NO_3 \cdot HCl$: C, 61.63; H, 7.11; N, 4.49; Cl, 11.37. Found: C, 61.16; H, 7.02; N, 4.44; Cl, 11.55.

Tropine *p*-nitrobenzoate hydrochloride, m.p. 278° dec.

Anal.—Calcd. for $C_{15}H_{18}N_2O_4 \cdot HCl$: C, 55.14; H, 5.86; N, 8.57; Cl, 10.85. Found: C, 55.22; H, 5.84; N, 8.57; Cl, 11.11.

Atropine ethylbromide and atropine benzylchloride were prepared by refluxing atropine base with an excess of ethyl bromide or benzyl chloride in a vessel fitted with an air condenser. The reaction mixture was cooled and allowed to stand for 24 hours. The appropriate ester was recrystallized from anhydrous ether and dried at 100°.

Atropine ethylbromide, m.p. 176° dec.

Anal.—Calcd. for $C_{19}H_{28}NO_3Br$: C, 57.30; H, 7.10; N, 3.50; Br, 20.06. Found: C, 57.27; H, 6.90; N, 3.45; Br, 20.03.

Atropine benzylchloride, m.p. 220–221°.

Anal.—Calcd. for $C_{24}H_{30}NO_3Cl$: C, 69.30; H, 7.27; N, 3.37; Cl, 8.52. Found: C, 69.15; H, 7.30; N, 3.34; Cl, 8.59.

Buffer System.—To maintain hydroxyl ion concentration essentially constant at low pH values, the buffer solutions shown in Table I were used. For high pH, $Ba(OH)_2$ solution was used. Since the concentration of ester was kept low relative to the $Ba(OH)_2$ concentration, the pH remained

TABLE II.—

Compd.	$K_b \times 10^5$	Activation Energy Kcal./mole	Log Frequency Factor	Rate Constant L./mole/sec., 30°C.
Reaction 1				k_1
Tropine phenylacetate	7.4	11.3	5.41	1.8×10^{-3}
Tropine phenoxyacetate	4.7	8.8	5.43	0.11
Tropine *p*-nitrobenzoate	0.2	9.8	5.71	4.7×10^{-2}
Nor-atropine	11.7	7.1	3.07	8.8×10^{-3}
Atropine[a]	7.3	7.7	3.54	9.8×10^{-3}
Homatropine[a]	7.6	12.3	7.62	5.2×10^{-2}
Reaction 2				k_2
Tropine phenylacetate	...	5.4	3.00	0.13
Tropine phenoxyacetate	...	7.9	5.89	1.52
Tropine *p*-nitrobenzoate	...	11.0	7.98	1.05
Nor-atropine	...	6.9	4.59	0.42
Atropine[a]	...	12.0	8.54	0.25
Homatropine[a]	...	11.4	8.72	2.05
Quaternary Esters				k
Atropine methylbromide[a]	...	14.0	9.82	0.54
Atropine ethylbromide	...	10.7	7.38	0.47
Atropine benzylchloride	...	15.8	11.43	1.01
Homatropine methylbromide[a]	...	13.0	9.74	2.34

[a] Calculated from literature data (1–3).

essentially constant during the course of the reaction.

Procedure.—The desired quantity of buffer solution was placed in a thermostatically controlled bath at the desired temperature and allowed to reach temperature equilibrium. One milliliter of a solution containing sufficient ester to give the reaction mixture a concentration of approximately 1 mg./ml. was added and mixed by inverting the flask several times. The first sample of the reaction mixture withdrawn immediately was designated as the "0" minute sample and analyzed for residual ester. Progress of the reaction was followed by the withdrawal of samples at suitable time intervals and determination of residual ester concentration.

For nor-atropine, the method reported in the literature for atropine (1) and homatropine (2) was followed. For tropine phenylacetate, tropine phenoxyacetate, and tropine *p*-nitrobenzoate, carbon tetrachloride was used for extraction because extraction was incomplete with chloroform as the solvent.

Second-order reaction rates of the quaternary salts, atropine ethylbromide, and atropine benzylchloride were followed by indicator photometry as described by Patel and Lemberger (3), using "Alizarin yellow R" solution for determining OH^- ions. It was found that the indicator was very sensitive to atmospheric oxidation; therefore 0.003 *M* sodium sulfite was added to the 0.00655 *M* $Ba(OH)_2$ solution, and the $BaSO_3$ formed acted as an antioxidant. The precipitated excess $BaSO_3$ was filtered off and the concentration of the hydroxyl ions was determined by titration.

RESULTS

Influence of Ester Concentration.—At constant temperature and hydroxyl ion concentration, the rate of hydrolysis of nor-atropine, tropine phenylacetate, tropine phenoxyacetate, and tropine *p*-nitrobenzoate was observed to be first-order with respect to ester in agreement with earlier studies (1, 2).

Influence of Hydroxyl Ion Concentration.—The effect of hydroxyl ion concentration on the rate of hydrolysis of nor-atropine (expressed in terms of half life in minutes of the ester) is illustrated in Fig. 1. Rate measurements were carried out for tropine phenylacetate, tropine phenoxyacetate, and tropine *p*-nitrobenzoate, one at high pH and one at low pH to obtain k_1 and k_2 for Reaction 1 and Reaction 2, respectively. K_b was determined from the pH of a half neutralized solution of the salt of the ester at 30°. These results are shown in Table II.

Temperature Dependency.—Figure 1 further verifies that above pH 12.0 the mechanism of

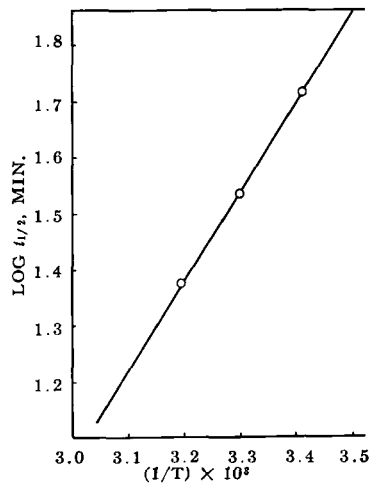

Fig. 2.—Temperature dependency of Reaction 1 for nor-atropine.

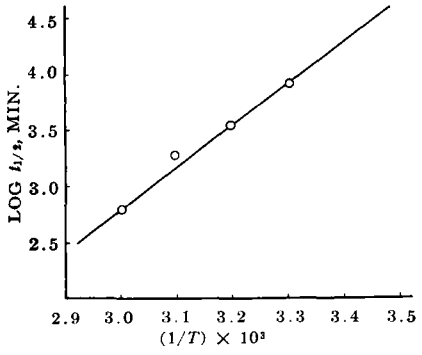

Fig. 3.—Temperature dependency of Reaction 2 for nor-atropine.

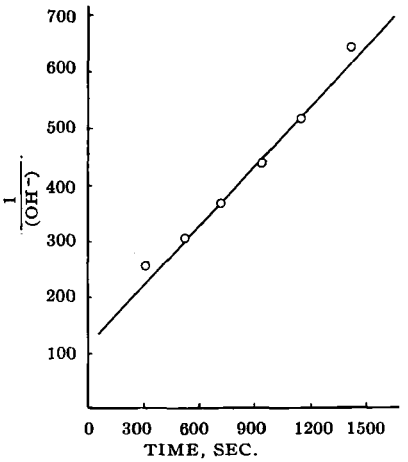

Fig. 4.—Typical bimolecular rate plot for the alkaline hydrolysis of atropine ethylbromide at 25.0°C. when alkali and ester are equal.

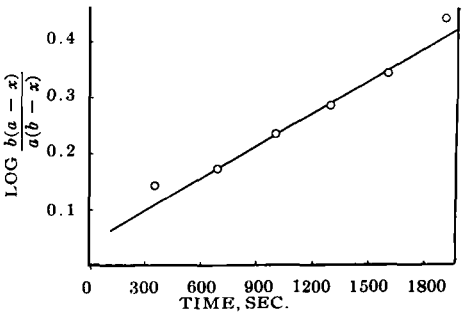

Fig. 5.—Typical bimolecular rate plot for the alkaline hydrolysis of atropine benzylchloride at 15.0°C. when alkali (*a*) and ester (*b*) are not equal.

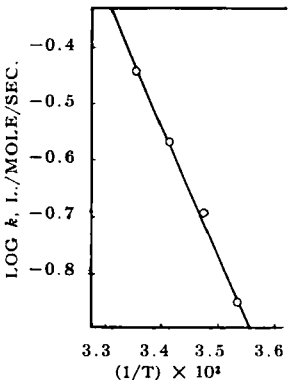

Fig. 6.—Arrhenius plot of the bimolecular rate constants for the alkaline hydrolysis of atropine ethyl bromide.

hydrolysis of these esters is that of Reaction 1, and that below pH 8.5 the mechanism is that of Reaction 2. Half lives were determined at different temperatures to study the temperature dependency of Reaction 1 and Reaction 2, respectively.

The logarithms of half life periods in minutes for these esters were plotted against the reciprocal of absolute temperatures and the straight line relationships obtained as illustrated in Figs. 2 and 3 indicate that the mechanism responsible for the ester hydrolysis is not altered by changes in temperature. The apparent activation energies were determined from the slopes of the plots and the frequency factors calculated from

$$k = se^{-\frac{E}{RT}}$$

The results are given in Table II.

Quaternary Salts.—The kinetics of the alkaline hydrolysis of the ester groups in atropine ethylbromide and atropine benzylchloride were determined by estimation of the slopes appropriate to the bimolecular rate expression

$$\frac{1}{a-x} = kt + \text{constant} \qquad \text{(Eq. 5)}$$

$$\log \frac{b}{a} \cdot \frac{(a-x)}{(b-x)} = \frac{kt\,(a-b)}{2.303} \qquad \text{(Eq. 6)}$$

$\frac{1}{a-x}$ was plotted against time when $a = b$ and $\log \frac{b}{a} \cdot \frac{(a-x)}{(b-x)}$ was plotted against time when $a \neq b$ where a and b are initial concentrations and $(a-x)$ and $(b-x)$ are concentrations at time intervals for the alkali and ester, respectively.

Hydroxyl ion concentration was calculated from the absorbance data.

Typical bimolecular rate plots for the hydrolysis of atropine ethylbromide and atropine benzylchloride are given in Figs. 4 and 5. Rate constants were determined at four different temperatures. Bimolecular rate constants are plotted against reciprocal of absolute temperature in Figs. 6 and 7. The rate constants at 30°, activation energies, and frequency factors are given in Table II.

DISCUSSION

Nor-atropine was studied extensively. The rather nice fit between the predicted relationship and the experimental points shown in Fig. 1 indicates that the hydrolytic Reactions 1 and 2 are mainly responsible for the hydrolysis of nor-atropine in aqueous solution. This behavior seems typical of the pattern of hydrolysis shown by other tropine esters reported previously (1–3). For this reason rate constants for hydrolysis of the other tropine esters reported here were determined at high (Reaction 1) and low (Reaction 2) pH only.

Of the esters listed in Table II tropine phenylacetate was observed to have the lowest hydrolytic rate for both Reactions 1 and 2. For convenience, hydrolysis rates of the other esters are compared to this compound. Thus, in Reaction 1 both atropine and nor-atropine hydrolyze approximately

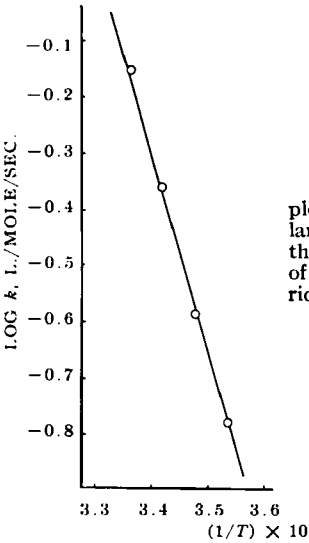

Fig. 7.—Arrhenius plot of the bimolecular rate constants for the alkaline hydrolysis of atropine benzylchloride.

five times faster than tropine phenylacetate; in Reaction 2 they are two and three times faster, respectively. This seems to indicate that replacement of one hydrogen on the nitrogen of nor-atropine with a methyl group to form atropine has virtually no effect on the rate of ester hydrolysis. With both these esters it is possible that the hydroxyl group in the acid moiety is responsible for an inductive effect on the carbonyl carbon. If this is true, the increase in positive character of the carbonyl carbon would facilitate hydrolysis of nor-atropine and atropine to account for the greater rate of hydrolysis relative to tropine phenylacetate.

In Reaction 1 tropine *p*-nitrobenzoate hydrolyzes approximately 25 times faster than tropine phenylacetate and in Reaction 2 eight times faster. The nitro group attached to the ester through the benzene ring seems to play a significant role in influencing hydrolytic rate. This has previously been observed (7, 8) and been attributed to the strong electron withdrawing tendency of the *p*-nitrobenzoyl group

TABLE III.—

Acid	Dissociation Constant, 25°C.	pKa
Phenylacetic	4.88×10^{-5}	4.31
Phenoxyacetic	5.56×10^{-5}	4.25
Tropic	7.50×10^{-5}	4.12
p-Nitrobenzoic	3.48×10^{-4}	3.46
Mandelic	4.29×10^{-4}	3.37

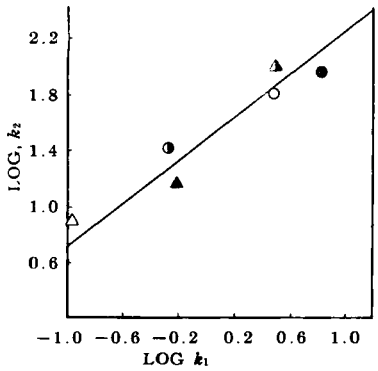

Fig. 8.—Plot showing the relationship of k_1 and k_2 of Reaction 1 and Reaction 2 for the following compounds at 30°C.: △, tropine phenylacetate; ▲, atropine; ◑, nor-atropine; ○, tropine *p*-nitrobenzoate; ⧋, homatropine; ●, tropine phenoxyacetate.

and the consequent formation of a highly positive center on the carbonyl carbon.

Homatropine is seen to hydrolyze 30 times faster than tropine phenylacetate by Reaction 1 and 15 times faster in the protonated form. This further confirms the inductive effect of the α-hydroxyl group in enhancing the positive character of the carbonyl carbon atom resulting in acceleration of the nucleophilic attack by hydroxide ion as reported previously (2).

As might be expected, if inductive effects are significant, the relative rates of hydrolysis of these esters should parallel the dissociation constants of the respective acids. Table III shows that this is mostly true. An exception is noted in the case of tropine phenoxyacetate whose rate of hydrolysis is 60 times faster by Reaction 1 and 11 times faster by Reaction 2 than the reference ester. These rates seem unusually high, considering that the dissociation constants of the acids are almost equal. It is possible that some other factor such as a steric effect may be involved, thus causing a greater hydrolytic rate than anticipated.

These observations are also apparent from the plot of log k_1 against log k_2 shown in Fig. 8. It can be seen that as polarity of the acid increases, the rate constants k_1 and k_2 increase. Similar observations have been made by Roberts and Moreland (9) in which they studied the electrical effects of substituted groups in saturated systems. They found that as the polarity of the acid increased, the effect of hydrolysis of its esters increased. It is also evident from Fig. 8 that the effect of polarity is more predominant in case of Reaction 1 than in Reaction 2, which would indicate that inductive effects are more prominent in hydrolysis of the base form of the ester.

The bimolecular rate of reaction of the quaternary nitrogen esters is of the same order of magnitude as the rate for the protonated form. Previous authors (3, 10) attribute this to the positive charge atmosphere which attracts hydroxyl ions to the vicinity of the carbonyl carbon in esters of esters of this type. Of the quaternary salts atropine methylbromide and atropine ethylbromide hydrolyze at approximately the same rate. There is some question whether the increased rate observed in atropine benzylchloride is truly significant. If so, it could perhaps be attributed to a steric facilitation by the relatively large benzyl group.

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Quantitative Determination of Morphine in Opium by Gas-Liquid Chromatography

By E. BROCHMANN-HANSSEN and A. BAERHEIM SVENDSEN†

Although morphine may be gas chromatographed as such, its phenolic properties cause adsorption which produces tailing and makes quantitative work impossible. This difficulty was overcome by converting morphine to its trimethylsilyl ether which gave a symmetrical elution peak suitable for quantitative estimation. The alkaloids of opium were extracted and morphine separated from nonphenolic alkaloids by ion-exchange resins. The morphine fraction was allowed to react with hexamethyldisilazane, tetraphenylethylene was added as the internal standard, and the solution was gas chromatographed on a column of silicone rubber SE-30.

DURING THE PAST few years, the gas chromatographic method has become increasingly important for analysis of high molecular weight compounds of biological origin. In 1960 Lloyd, *et al.* (1), gas chromatographed 45 different alkaloids, including the major alkaloids of opium at temperatures slightly above 200°. Eddy, *et al.* (2), used gas chromatography for the determination of the origin of opium based on the peak height ratios of several of the major alkaloids.

When working with alkaloids on low-loaded columns, tailing can often cause considerable difficulties. The support material will contain active spots which will not be sufficiently covered by the stationary liquid to prevent adsorptive effects. Adsorption can be reduced to a great extent if the support material is washed with acid and alkali and treated with dichlorodimethylsilane (3), hexamethyldisilazane (4), and/or a surface-active agent (4–6). Phenolic alkaloids, such as morphine, are difficult to gas chromatograph even on a treated support. If adsorption takes place, it will affect the retention time as well as the height and area of the emerging peak, and quantitative work becomes impossible. This difficulty may be overcome by converting the phenolic hydroxyl groups to esters or ethers. Hexamethyldisilazane is excellently suited for this purpose (7). At room temperature it produces trimethylsilyl ethers in quantitative yield.

Perhaps the most crucial step in a quantitative determination of morphine in opium is the extraction. Strongly acidic cation-exchange resins have been shown to effect rapid and efficient extraction of alkaloidal crude drugs including opium (8–11). The total alkaloids thus extracted may be effectively purified and phenolic and nonphenolic alkaloids separated by means of a strongly basic anion-exchange resin (9, 10). Such purification prior to the gas chromatographic analysis increases the life of the column, the specificity of the method, and simplifies the quantitative evaluations.

During the development of the method described below, two internal standards were used, tetraphenylethylene and laudanosine, which are eluted on either side of morphine 3,6-di-trimethylsilyl ether (Fig. 1). Both standards, however, give the same results. Tetraphenylethylene is readily available and is stable at the experimental conditions of this method. Most of our samples were analyzed with this substance alone as the internal standard.

EXPERIMENTAL

Twelve opium samples were analyzed. Eleven of these were authenticated samples obtained through the United Nations' Division of Narcotic Drugs.

Preparation of the Sample.—Two-hundred milligrams of finely powdered opium was triturated in a glass mortar with 1 ml. of hot water. Gradually, 10 ml. of hot water was added while stirring. The stirring was continued for 5 minutes, and the aqueous extract was decanted into an extraction

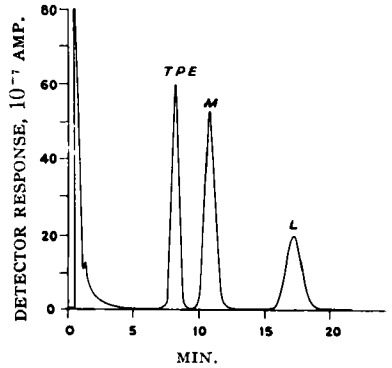

Fig. 1.—Gas chromatogram of morphine (M) isolated from a sample of opium by ion-exchange techniques. Tetraphenylethylene (TPE) and laudanosine (L) are added as internal standards.

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tube (8) containing 3 Gm. of an analytical grade of Dowex 50-X_2, 50–100 mesh. The residue was extracted twice more with 10 ml. of hot water each time and the extracts added to the extraction tube. The opium residue was then washed quantitatively into the tube with enough hot water to give a volume of about 50 ml. The tube was shaken mechanically for 15 minutes. The liquid was allowed to drain slowly from the extraction tube, and the resin in the tube was washed with about 50 ml. of water.

The alkaloids on the resin were eluted slowly with 100 ml. 1 *N* methanolic ammonia (68 ml. of concentrated ammonia, 700 ml. of reagent grade methanol, and enough water to make 1000 ml.). The eluate was allowed to pass through a 1.2 cm. × 30-cm. column of an analytical grade of Dowex 1-X_1, 50–100 mesh. The resin was previously activated with 4 *N* sodium hydroxide and washed free of excess alkali with distilled water. The cationic resin was washed with 50 ml. of 70% methanol, and the washings allowed to flow through the anionic-exchange column. Finally, the anionic-exchange resin was washed with water until the washings were neutral to phenolphthalein, and morphine was eluted slowly with 100 ml. of 0.5 *N* acetic acid. The morphine eluate was collected in a 250-ml. round-bottomed flask and evaporated to dryness in a rotating vacuum evaporator. To remove completely the last traces of water and acetic acid, 10 ml. of absolute ethanol was added and removed under reduced pressure. This was repeated a second time. Thirty-five milligrams of tetraphenylethylene was added to the flask, and the internal standard and alkaloidal residue were dissolved in 2 ml. of pyridine, which had been redistilled over phosphorus pentoxide. One milliliter of hexamethyldisilazane was added to the solution, the flask was stoppered with a glass stopper, and set aside for 24 hours.

Gas Chromatography.—The instrument used for this work was a Barber-Colman model 15 gas chromatograph equipped with an argon ionization detector containing 56 μc. of Ra-226. The column was a glass U-tube, 4 ft. long and 4 mm. in inside diameter. The solid support was Gas-Chrom P, 60–80 mesh, which was washed with concentrated hydrochloric acid, methanolic potassium hydroxide, dried, and treated with hexamethyldisilazane (4). The dried and siliconized support was coated with 0.1% of polyethylene glycol 9000 and, finally, with 4% of silicone rubber, SE-30 (7). The column temperature was maintained at 183°, the injection port (flash heater) temperature was 280°, and the detector cell temperature was 240°. The gas pressure at the inlet was 20 lb., producing a gas flow of 250 ml. per minute. The solutions to be analyzed were injected with a Hamilton microliter syringe, the usual sample size being about 3 μl., corresponding to about 20 to 30 mcg. of morphine. The instrument was operated at submaximal sensitivity to reduce the noise level and gain linearity of detector response. The peak areas were measured with a planimeter, each area reading being based on the average obtained from the cumulative value of ten consecutive tracings. The area ratio of morphine (M) to tetraphenylethylene (TPE) was determined, the corresponding weight ratio obtained from a standard curve, and the percentage of morphine in the sample calculated.

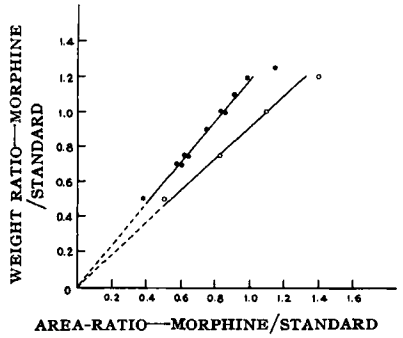

Fig. 2.—Standard curves for morphine with two internal standards. Key: ●, morphine/tetraphenylethylene; ○, morphine/laudanosine.

Standard Curve.—In each of several glass vials were placed 20 mg. of tetraphenylethylene, 20 mg. of laudanosine base,[1] and known amounts of anhydrous morphine base ranging from 10 to 25 mg. The substances were dissolved in 2 ml. of dry pyridine; 1 ml. of hexamethyldisilazane was added. The vials were stoppered with polyethylene stoppers and set aside at room temperature. The reaction appeared to be complete in 16 to 18 hours. An additional reaction time of several days produced no change in the result. The solutions were gas chromatographed, and the weight ratio of morphine to the internal standard was plotted against the corresponding ratio for the peak areas. A straight line was obtained over a range sufficiently great to include all samples encountered (Fig. 2).

The linear range of the argon ionization detector is narrower than that of most other detectors used in gas chromatography (12). However, by adjusting the amount of internal standard added to the unknown it is always possible to work within the linear part of the curve. Although our standard curve appeared to be very reproducible, we deemed it desirable to check it with new standard solutions every day that unknown samples were gas chromatographed.

RESULTS AND DISCUSSION

The contents of morphine in the opium samples analyzed are recorded in Table I. The results are compared with results obtained by other workers using different methods. The reproducibility of the method is satisfactory. Based on a total of 40 determinations of ten different opium samples, the precision, calculated as the standard deviation, was 0.15%.

It is well known that the lime method used by the U.S.P. for determination of morphine in opium does not give results which are absolutely correct. One reason for this is that the crystallization of morphine is incomplete so that a certain amount is lost in the mother liquor (16, 17). It has also been shown that the morphine crystals are contaminated with other opium alkaloids (17). Mannich's method (18), which is based on precipitation of the dinitrophenyl ether of morphine, is considered by many workers to give more accurate results (14, 17, 19, 20).

Fulton (21, 22) has described certain phenolic opium alkaloids which follow morphine very closely

[1] We are indebted to Mallinckrodt Chemical Works, St. Louis, Mo., for supplying us with this alkaloid.

TABLE I.—MORPHINE CONTENT IN OPIUM SAMPLE

Opium Sample	Morphine Content, %—G.L.C. Method[a]—Value Determined	Calcd. to Anhydr. Basis	Other Methods
U.S.P.	11.4	...	10.5[b]
UN2A	14.0	14.9	13.5[c]
UN15	16.5	17.5	16.1[d]
UN38G	18.5	19.7	17.0,[c] 20.3[d]
UN137A	15.1	16.1	13.8[c]
UN25A	18.3	19.5	18.1,[c] 19.07[d]
UNE529	13.8	14.6	13.8[c]
UNE531	11.0	11.7	12.0[c]
UN265	12.8	13.6	13.1[c]
UNE612	15.5	16.3	15.5[c]
UNE627	12.4	13.1	11.0[c]
UNE631	12.2	13.2	12.0[c]

[a] Average values based on two or more determinations. [b] Opium assay U.S.P. XVI (13). [c] Modified Mannich method (14); sample analyzed without drying. [d] United Nations' Secretariat (15); calculated to anhydrous basis.

TABLE II.—GAS CHROMATOGRAPHIC DETERMINATION OF MORPHINE IN OPIUM BEFORE AND AFTER PURIFICATION *via* DICHLOROACETIC ACID

Opium Sample	Morphine, %—No Special Treatment	Purification *via* $CHCl_2COOH$
UN38G	18.6	18.6
	18.4	18.4
UNE265	12.8	12.6
UNE612	15.4	15.4
	15.6	15.5

in many of its reactions and extractions. They are present in amounts up to 1.5% and may lead to high results if the analytical procedure is not sufficiently specific. It is possible to remove them by extracting the acidified morphine fraction with chloroform in the presence of dichloroacetic acid (15). These alkaloids do not interfere in the gas chromatographic determination of morphine. Table II shows that inclusion of an extra purification step *via* dichloroacetic acid does not change the results.

The generally higher results obtained by the gas chromatographic method than by the other procedures referred to in Table I are probably due to a more complete extraction of opium by the ion-exchange resin and a more quantitative recovery during the purification steps.

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2-Aminobenzenethiol Derivatives as Potential Psychotherapeutic Agents

By KARL A. NIEFORTH

A structural similarity between reserpine and chlorpromazine is described and a series of derivatives of 2-aminobenzenethiol designed to match the similarity is synthesized and tested for pharmacologic activity.

THE APPARENT tranquilizing activity of reserpine and chlorpromazine is qualitatively the same, although the two compounds are quite different structurally. It is postulated that the two compounds act by two pharmacologically distinct mechanisms—reserpine by trophotropic stimulation and chlorpromazine by ergotropic inhibition according to the terminology of Hess (1).

With the use of molecular models, a structural similarity may be seen between the two compounds (see Fig. 1). The diagram represents the positional relationships of three atoms in the two compounds and shows the location of an aromatic structure. Position *A* represents the phenolic oxygen of reserpine or the sulfur of chlorpromazine; position *B*, the aromatic nitrogen of each compound; and position *C*, the aliphatic nitrogen of each compound. It must be kept in mind that the positions also could represent any bioisosteric modification. A similarity of

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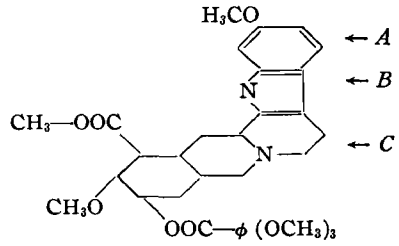
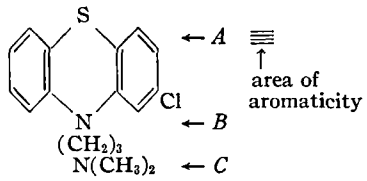


Reserpine



Chlorpromazine

Fig. 1.—Structural similarity of reserpine and chlorpromazine as obtained with Stuart-Briegleb molecular models.

the compounds which is difficult to express by a diagram but readily visible by the use of molecular models is the proximity in space occupied by substituents on position 2 of promazine and position 16 of reserpine. This may explain why substituents on position 2 of promazine have a greater effect on activity than do similar substituents on other positions of the phenothiazine structure.

It has been proposed that the sites involved in the activity of reserpine and chlorpromazine are specific for the corresponding drugs because of the apparent dissimilarity of structures. On the basis of the similarity shown in Fig. 1, it seems that the two sites involved in CNS activity may be closely related in physical characteristics or that only one site may be involved, the latter being more probable. This particular arrangement of atoms does not necessarily result in reserpine-chlorpromazine type CNS depression as illustrated by the stimulant imipramine (2). In some instances, activity may be found in compounds possessing only a part of the total arrangement such as the depressant tetrabenazine (3).

The compounds in this series (Table II) were designed to have a hybrid reserpine-chlorpromazine activity. Activity of this type would suggest the existence of a single site with differences which are observed in the pharmacodynamic actions accounted for by secondary effects of the drugs. Figure 2 shows the reaction scheme which was suitable for the synthesis of the test compounds.

The circuitous route to compound VII from compound II was necessitated by the difficulty encountered in separating the products of methylation of compound II. Methyl 2-acetamidophenyl sulfide and its N-methyl analog decomposed upon distillation and could not be used to form methyl 2-methylaminophenyl sulfide without tedious recrystallizations. For this reason, methyl 2-benzenesulfonamidophenyl sulfide and its N-methyl derivative were used as intermediates. These two compounds could be separated and purified easily by their solubilities in alkaline solution and were prepared in good yields. The sulfonamide procedure could have been used for the preparation of all of the alkyl 2-methylaminophenyl sulfides (Table I), but purification of the acetyl derivatives was more rapid than was recrystallization of the sulfonamide derivatives.

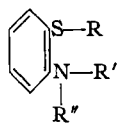
EXPERIMENTAL

Alkyl 2-Aminophenyl Sulfides (II).—These compounds were synthesized by well documented procedures in good yields. Running the reaction under nitrogen improved its appearance but had little effect on the yields.

Alkyl 2-Acetamidophenyl Sulfides (III).—One mole of alkyl 2-aminophenyl sulfide and 1 mole of pyridine were dissolved in 500 ml. of ether and 1 mole of acetyl chloride added over the period of 1 hour. The mixture was stirred for 1 hour, poured into diluted hydrochloric acid, extracted with ether, and distilled at reduced pressure.

Alkyl 2-(N-methyl)-acetamidophenyl Sulfides (IV).—Alkyl 2-acetamidophenyl sulfide (0.8 mole) was added to a sodium dispersion (1.0 mole in 300

TABLE I.—SUBSTITUTED 2-AMINOBENZENETHIOLS



R	R'	R''	Yield	B.p./Pressure M.p., °C.[a]	Formula	Nitrogen, %[d] Calcd.	Found
CH_3	H	H	94	112/6.5 mm.	C_7H_9NS		[b]
CH_3	CO—CH_3[c]	H	92	96–97	$C_9H_{10}NOS$	7.72	7.50
CH_3	SO_2—C_6H_6[c]	H	95	80–82	$C_{13}H_{14}NO_2S_2$	5.01	4.67
CH_3	SO_2—C_6H_6[c]	CH_3	92	115–116	$C_{14}H_{16}NO_2S_2$	4.79	4.58
CH_3	H	CH_3	57	98/4.7 mm.	$C_8H_{11}NS$	9.14	9.12
C_2H_5	H	H	93	84/1.3 mm.	$C_8H_{11}NS$		[b]
C_2H_5	CO—CH_3	H	77	164/6 mm.	$C_{10}H_{13}NOS$	7.17	7.01
C_2H_5	CO—CH_3	CH_3	98	121/0.42 mm.	$C_{11}H_{15}NOS$	6.75	6.62
C_2H_5	H	CH_3	64	121/7.5 mm.	$C_9H_{13}NS$	8.42	8.44
$CH(CH_3)_2$	H	H	89	87/1.0 mm.	$C_9H_{13}NS$		[b]
$CH(CH_3)_2$	CO—CH_3	H	85	161/6.5 mm.	$C_{11}H_{15}NOS$	6.75	6.82
$CH(CH_3)_2$	CO—CH_3	CH_3	77	171/6.5 mm.	$C_{12}H_{17}NOS$	6.32	6.30
$CH(CH_3)_2$	H	CH_3	73	126/8.0 mm.	$C_{10}H_{15}NS$	7.81	7.78

[a] Melting points were taken on a Thomas melting point apparatus and are uncorrected. [b] Prepared by Foster, D., and Reid, E., *J. Am. Chem. Soc.*, **46**, 1941(1924). [c] Recrystallized from 95% ethanol. [d] Microanalyses were carried out by Alfred Bernhardt, Mikroanalytisches Laboratorium, Max-Planck-Institut, Mulheim (Ruhr), Germany.

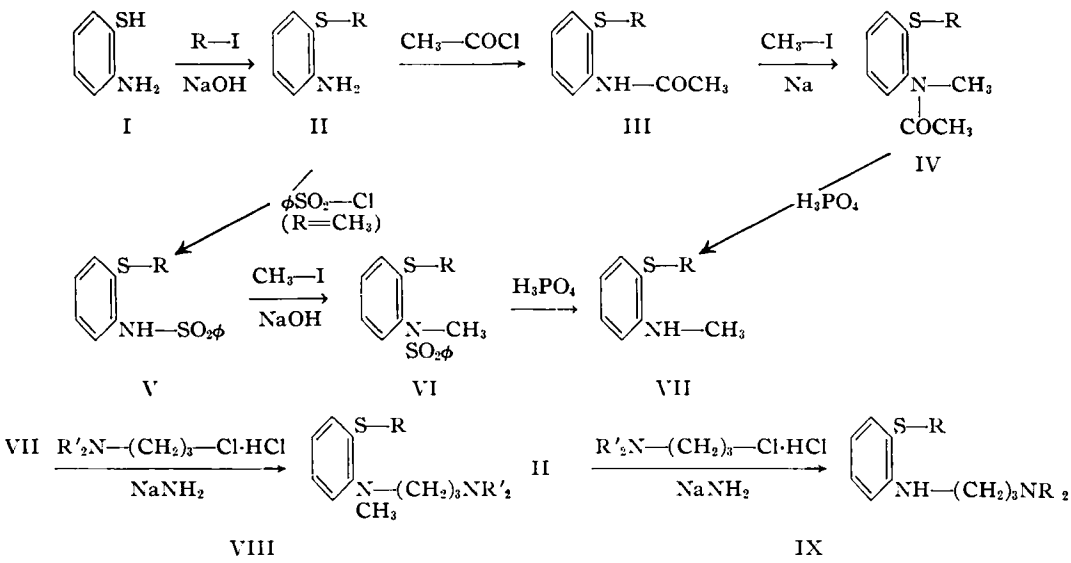


Fig. 2.—Synthetic procedure.

ml. of toluene) and stirred on a steam bath for 10 hours. After cooling to room temperature, methyl iodide (1.0 mole) was added with stirring over a period of 2 hours. The reaction was stirred for an additional hour without heating. The solvent was removed and 20 ml. of ethanol added to destroy excess sodium. An aqueous suspension of the residue was extracted with ether and the ethereal solution distilled at reduced pressure.

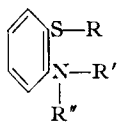
Methyl 2-Benzenesulfonamidophenyl Sulfide (V).—A mixture of methyl 2-aminophenyl sulfide (1.0 mole) and pyridine (1.0 mole) was dissolved in 500 ml. of ether. Benzenesulfonyl chloride (1.0 mole) was added with stirring over a period of 30 minutes. The reaction was heated on a steam bath for 30 minutes, after which it was extracted with dilute hydrochloric acid. The ether was evaporated and the residue solidified by cooling.

Methyl 2-(N-methyl)-benzenesulfonamidophenyl Sulfide (VI).—A solution of methyl 2-benzenesulfonamidophenyl sulfide (0.2 mole) was prepared by dissolving it in 275 ml. of 5% sodium hydroxide. Methyl iodide (0.24 mole) was added during 30 minutes with stirring. The reaction was then refluxed for 1 hour, cooled to room temperature, and filtered. The solid product was treated with 20% sodium hydroxide to remove any starting material and then recrystallized from 95% ethanol.

Alkyl 2-Methylaminophenyl Sulfides (VII).—These compounds could be prepared from either methyl 2-(N-methyl)-benzenesulfonamidophenyl sulfide (VI) or alkyl 2-(N-methyl)-acetamidophenyl sulfides (IV) in the same manner. The reactant was heated at 150° for 18 hours with an excess of orthophosphoric acid. The reaction was cooled and carefully neutralized with dilute sodium hydroxide solution. The mixture was extracted with toluene or benzene while still warm before the precipitation of sodium phosphate. The solvent was removed and the residue distilled at reduced pressure.

Alkyl N-(N'-dialkylaminoalkyl)-N-methylaminophenyl Sulfides (IX).—Alkyl 2-methylaminophenyl sulfide (0.09 mole) was heated in 200 ml. of toluene with sodium amide (0.2 mole) for 24 hours. Dialkylaminoalkylchloride hydrochloride (0.08 mole) was added in one addition; the reaction was heated and stirred for an additional 24 hours. The toluene was

Table II.—Alkyl 2-Dialkylaminoalkylaminophenyl Sulfides



R	R'	R"	B.P./Pressure	Yield	Nitrogen, % Calcd.	Nitrogen, % Found
CH_3	CH_3	$CH_2—CH_2—CH_2—N(CH_3)_2$	144/1.7 mm.	70	11.75	11.53
CH_3	CH_3	$CH_2—CH_2—CH_2—N(C_2H_5)_2$	156/2.2 mm.	89	10.51	10.55
C_2H_5	CH_3	$CH_2—CH_2—N(CH_3)_2$	135/1.75 mm.	39	11.75	11.47
C_2H_5	CH_3	$CH_2—CH_2—CH_2—N(CH_3)_2$	140/2.0 mm.	92	11.09	10.93
C_2H_5	CH_3	$CH_2—CH_2—CH_2—N(C_2H_5)_2$	154/2.0 mm.	92	9.91	10.06
$CH(CH_3)_2$	CH_3	$CH_2—CH_2—CH_2—N(CH_3)_2$	142/2.0 mm.	68	10.51	10.31
$CH(CH_3)_2$	CH_3	$CH_2—CH_2—CH_2—N(C_2H_5)_2$	155/1.5 mm.	77	9.44	9.43
$CH(CH_3)_2$	H	$CH_2—CH_2—CH_2—N(C_2H_5)_2$	135/1.75 mm.	82	9.91	9.78

removed, and a small amount of ethanol was added to destroy residual sodium amide. The ethanol was removed, the residue was suspended in a saturated solution of potassium carbonate, and extracted with ether. The ether was removed and the residue distilled at reduced pressure. This is a modification of a procedure reported by Huttrer (4).

Isopropyl N-(N'-Diethylaminopropyl)-aminophenyl Sulfide (VIII).—This compound was prepared in the same manner as compound IX. Isopropyl 2-methylaminophenyl sulfide (0.13 mole) was reacted with sodium amide (0.25 mole) in 200 ml. of toluene. After heating the mixture for 24 hours, 3-diethylaminopropylchloride hydrochloride was added and heated for 24 hours. The product was isolated in the manner described above in a yield of 82%. Compounds of this type have been previously reported (5).

These compounds were screened for gross pharmacologic activity using the Hippocratic screen method (6). Biphasic activity was exhibited by an initial increase in motor activity accompanied by evidence of disorientation and stereotypy in the form of head shaking, chewing motions, and prancing of the forelimbs. This was followed by ataxia and decreased motor activity 1 hour after intraperitoneal injection. One compound, isopropyl N-(N'-diethylaminopropyl)-aminophenyl sulfide exhibited only motor activity depression without an initial increase.[1]

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(2) Ditfurth, H., *Med. Exptl.*, **2**, 147(1960).
(3) Cahn, J., and Herold, M., *Psychiat. Neurol.*, **140**, 210 (1960).
(4) Huttrer, C. P., Djerassi, C., Beears, W. L., Mayer, R. L., and Scholz, C. R., *J. Am. Chem. Soc.*, **68**, 1999(1946).
(5) Davis, C. S., Jenkins, G. L., Knevel, A. M., and Paget, C., THIS JOURNAL, **51**, 841(1962).
(6) Malone, M., and Robichaud, R., *Lloydia*, **25**, 320 (1962).

[1] Preliminary pharmacological data were provided by Dr. Marvin M. Malone and Mr. Roger C. Robichaud, Division of Pharmacology, Pharmacy Research Institute, University of Connecticut, Storrs.

Effect of Certain Tablet Formulation Factors on Dissolution Rate of the Active Ingredient III

Tablet Lubricants

By GERHARD LEVY and ROBERT H. GUMTOW

A hydrophobic tablet lubricant (magnesium stearate) has been found to retard the dissolution of salicylic acid from model compressed tablets, while a water-soluble, surface-active lubricant (sodium lauryl sulfate) enhanced markedly the dissolution rate. Experiments with nondisintegrating disks indicate that the more commonly used hydrophobic lubricants (magnesium stearate, aluminum stearate, stearic acid, talc) decrease the effective drug-solvent interfacial area and thereby decrease the rate of dissolution of the drug, while water-soluble lubricants (sodium oleate, sodium lauryl sulfate) do not have this effect. The dissolution rate enhancing effect of sodium lauryl sulfate (in the case of conventional tablets) is not due to any modification of microenvironmental pH or solubilization by micelles, but rather to the better penetration of solvent into tablets and their component granules and the resulting greater availability of drug surface.

THE EFFECT of formulation and processing factors on the dissolution rate of active ingredients of compressed tablets has been the subject of extended investigation in this laboratory (1, 2). The present report deals with the effects of tablet lubricants and the mechanisms by which they may modify the dissolution rate of pharmaceuticals contained in tablets.

The more commonly used tablet lubricants are hydrophobic substances. Their water-repellent effect is evidenced by their tendency to increase markedly the disintegration time of tablets (3–5). When lubricants are added to a tablet granulation, they form a coat around individual granules which remains more or less intact during the process of tablet compression (3). Interference by these agents with the dissolution of drugs in aqueous media is therefore a likely possibility.

An extensive study of currently used and potentially useful tablet lubricants by Strickland, *et al.* (6), revealed that a few water-soluble substances are effective lubricants. Presumably, these substances, unlike hydrophobic lubricants, will not interfere with the dissolution of tablet ingredients. For this reason, both hydrophobic lubricants as well as certain water-soluble lu-

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TABLE I.—SPECIFICATIONS FOR EXPERIMENTAL TABLETS

Formula Designation	Granules: Compn.[a]	Granules: Precompression Pressure[b]	Granules: Size[c]	Tablets: Compn.	Tablets: Compression Pressure[b]
A	SA 60–80 mesh	2150	20–40	SA granules, 300 mg. Starch, 60 mg. Lubricant, 9 mg.	715
B	SA 60–80 mesh Starch, 10%	1430	20–40	SA Starch granules 300 mg. SA equiv. Starch, 15 mg. Lubricant, 9 mg.	715

[a] SA = Salicylic acid. [b] Kg./cm.^{2} [c] U.S.P. mesh size of granules used for preparation of tablets.

bricants were used in this study. They were selected on the basis of high R value[1] and ejection force lowering capability from among those evaluated by Strickland, *et al.* (6). The particular dissolution test for compressed tablets (beaker method) and the experimental tablet formulations used in this study were chosen for reasons that have been discussed previously (1, 2).

EXPERIMENTAL

Preparation of Tablets

Salicylic acid tablets were prepared according to a method described previously (2). Composition and processing conditions of the two experimental preparations are listed in Table I. All lubricants were passed through a 100-mesh sieve.

Dissolution Rate Determination for Tablets.—Dissolution rates were determined by the beaker method (7) and in one instance also by the oscillating tube method (1). For the latter procedure, the volume of dissolution medium was reduced to 300 ml.

Preparation of Disks

Salicylic acid powder was precompressed at 6000 lb. total force into 0.5-in. diam. slugs by a modified hydraulic press (Carver, model B). The slugs were crushed into granules with mortar and pestle, and the 20–40-mesh granule fraction was collected by sieving. Lubricant was added to the granules in a small plastic vial, and these were mixed gently but thoroughly by rotating the vial. The granulation was compressed into thin, nondisintegrating disks of $^1/_2$ in. diam. with the modified hydraulic press, using 20,000 lb. total force. In one instance, disks were prepared directly from 100-mesh salicylic acid powder and from a mixture of salicylic acid powder (100 mesh) and lubricant.

Dissolution Rate Determinations for Disks.—Dissolution rates were determined by the rotating disk (8, 9) and static disk (1) methods, using 0.1 *N* hydrochloric acid at 37° as the dissolution medium. Speed of rotation for the rotating disk experiments was 555 r.p.m. Except where otherwise indicated, all data shown in the figures represent the average of duplicate determinations.

RESULTS AND DISCUSSION

Studies with Compressed Tablets.—A commonly used and very effective hydrophobic lubricant (magnesium stearate) and a very effective (6) water-soluble lubricant (sodium lauryl sulfate) were used in these studies. Figure 1 shows the effect of these agents on the dissolution rate of salicylic acid in compressed tablets made from granules containing pure drug. Magnesium stearate decreased dissolution rate appreciably, while sodium lauryl sulfate had the opposite effect. Figures 2 and 3 show the effect of magnesium stearate and sodium lauryl sulfate, respectively, on the dissolution rate of salicylic acid tablets made from granules that contained salicylic acid and starch. Again, magnesium stearate decreased and sodium lauryl sulfate increased the dissolution rate of salicylic acid. Comparison of Fig. 1 with Fig. 3 shows that sodium lauryl sulfate had a greater enhancing effect when it was incorporated in tablets that were made from starch-containing, *i.e.*, disintegrating granules. The latter formulation was also tested by means of the oscillating tube method (compared with the

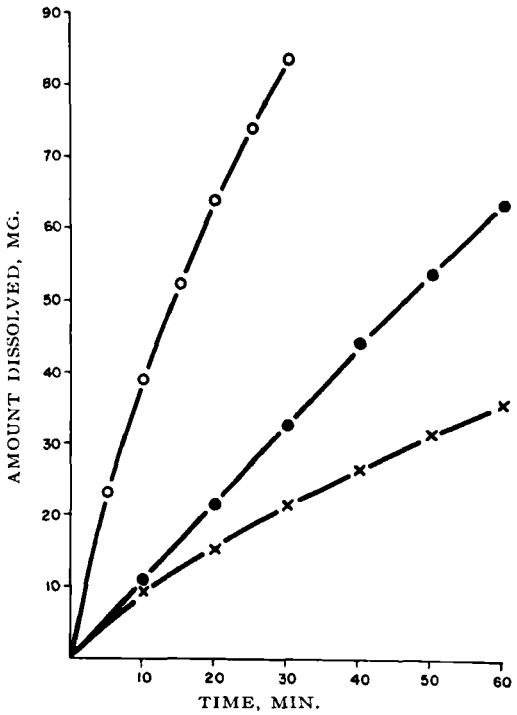

Fig. 1.—Effect of lubricant on dissolution rate of salicylic acid contained in compressed tablets (formula A). Key: ×, 3% magnesium stearate; ●, no lubricant; ○, 3% sodium lauryl sulfate. (Average of 10 tablets each).

[1] This is the ratio of lower to upper punch forces at maximum compression. A hypothetical perfect lubricant has a R value of unity (6).

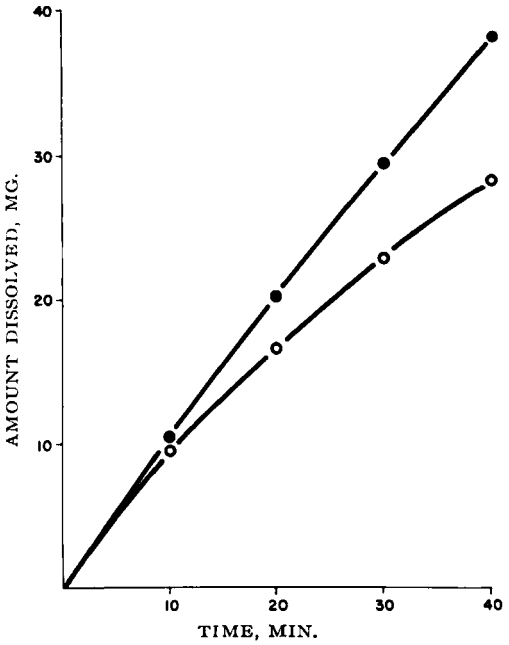

Fig. 2.—Effect of lubricant on dissolution rate of salicylic acid contained in compressed tablets (formula B). Key: O, 3% magnesium stearate; ●, no lubricant. (Average of five tablets each).

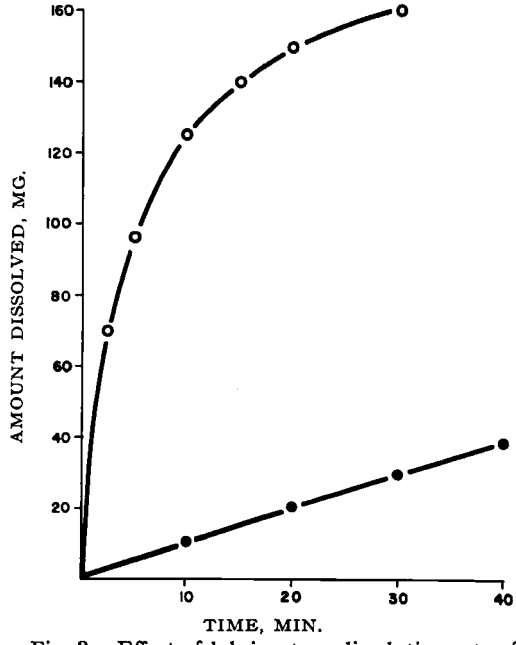

Fig. 3.—Effect of lubricant on dissolution rate of salicylic acid contained in compressed tablets (formula B). Key: O, 3% sodium lauryl sulfate; ●, no lubricant. (Average of five tablets each).

beaker method). The results of this experiment, shown in Fig. 4, though quantitatively different, are qualitatively similar to those obtained with the beaker method.

Cooper and Brecht (10) have found that incorporation of surfactants into tablet formulations was an effective means of reducing tablet disintegration time. Sodium lauryl sulfate had no significant effect in their experience, but this is probably due to their use of calcium lactate tablets as the test system. Sodium lauryl sulfate reacts with calcium ion to yield a water-insoluble compound. Ward and Trachtenberg (11) have found that sodium lauryl sulfate did reduce the disintegration time of tablets made from amphenidone and sulfadiazine, respectively.

The experimental results depicted in Figs. 1–4 parallel those obtained from studies of the effect of stearates (3–5) and surfactants (10, 11), respectively, on the disintegration rate of tablets. However, this does not mean that the effect of these lubricants on dissolution rate is mediated solely or even primarily by their modification of tablet disintegration rates. In fact, the experimental tablet preparations used in this study were formulated in a manner which resulted in their rapid disintegration in order to minimize the effect of differences in tablet disintegration rates.

In the case of sodium lauryl sulfate, the dissolution rate enhancing effect could be due in part to an increase in microenvironmental pH (2),[2] to solubilization by micelles (12), and to more complete wetting of the drug solids (12). The dissolution rate retarding effect of magnesium stearate could be partially the result of delayed granule disintegration and reduced contact between drug and solvent. To investigate most of these possibilities it was desirable to eliminate totally tablet disintegration rate as an experimental variable. This was accomplished by the use of nondisintegrating pellets (disks) made from pure drug granules and lubricant.

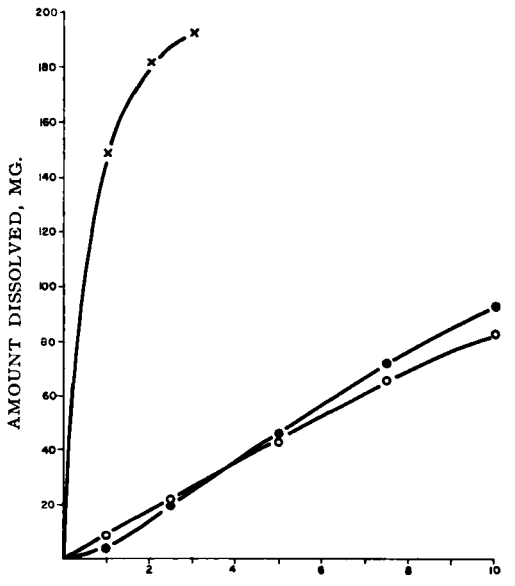

Fig. 4.—Effect of lubricant on dissolution rate of salicylic acid contained in compressed tablets (formula B) as determined by the oscillating tube method. Key: O, 3% magnesium stearate; ●, no lubricant; ×, 3% sodium lauryl sulfate. (Average of five tablets each).

[2] A slurry of sodium lauryl sulfate in 0.1 *N* hydrochloric acid at 37° has a pH of approximately 9.2.

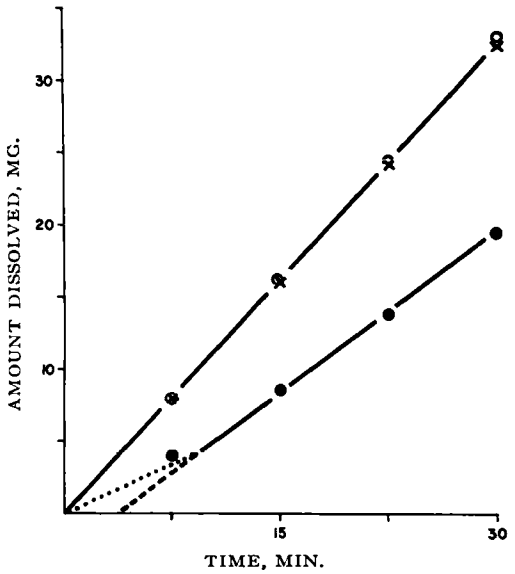

Fig. 5.—Effect of lubricant on the rate of dissolution of salicylic acid from rotating disks. Key: ●, 3% magnesium stearate; O, no lubricant; ×, 3% sodium lauryl sulfate; ---, extrapolation of experimental line; ..., line from Fig. 8. (Average of five disks each).

Studies with Nondisintegrating Disks.—As shown in Fig. 5, sodium lauryl sulfate had no effect on the dissolution rate of salicylic acid from the surface of nondisintegrating disks. This indicates that neither increased microenvironmental pH nor micellular solubilization are the cause for the dissolution rate enhancing effect of sodium lauryl sulfate observed in experiments with compressed tablets.

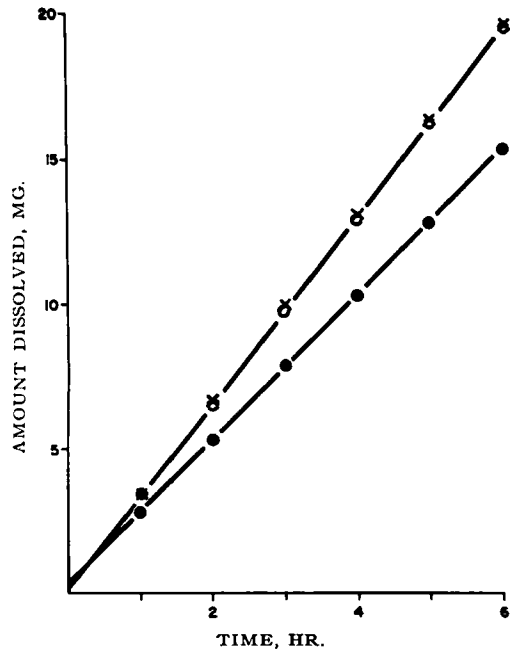

Fig. 6.—Effect of lubricant on the rate of dissolution of salicylic acid from static disks. Key: ●, 3% magnesium stearate; O, no lubricant; ×, 3% sodium lauryl sulfate.

The plot of amount dissolved *versus* time for disks containing magnesium stearate, also shown in Fig. 5, was somewhat unusual. Though linear (except for the first experimental point), it yielded a positive time intercept upon extrapolation. This suggested that magnesium stearate formed an impervious surface barrier which had to be removed before dissolution of drug could occur. If the surface of magnesium stearate–containing disks is, indeed, covered by an impervious barrier of the lubricant, then the apparent "lag-time" should be even greater under conditions where the mechanical effect of stirring is absent. However, when the dissolution rate was determined by the static disk method, there was no increase in apparent "lag-time," but rather, there was no apparent "lag-time" whatsoever (Fig. 6).

To determine whether the apparent "lag-time" found with rotating disks was real, experiments were carried out in which the dissolution medium was sampled every 2 minutes. This established that the apparent "lag-time" was because of an apparent increase in dissolution rate after about 5 mg. of drug had dissolved. Specifically, dissolution rate was constant until about 4 mg. of drug had dissolved, then gradually increased until it became constant once again after about 7 mg. had dissolved. The dotted line in Fig. 5 represents the experimentally determined initial dissolution rate of salicylic acid from magnesium stearate–containing disks. The subsequent change in apparent rate is seen readily.

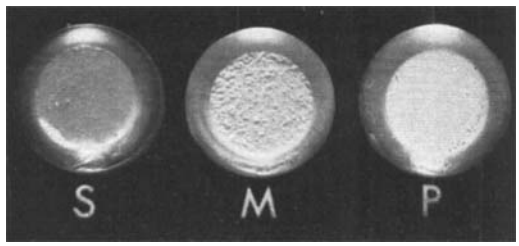

Fig. 7.—Surface of disks after dissolution of 10 mg. salicylic acid. Key: S = containing 3% sodium lauryl sulfate, M = containing 3% magnesium stearate, P = containing only pure salicylic acid.

These results may be explained on the basis of the changing surface characteristics of disks made from salicylic acid granules and magnesium stearate. The lubricant is confined to the surface and intergranular regions of the disks (3) and, as a result, the granules are surrounded by a water-insoluble matrix. As some of the salicylic acid dissolves, it leaves pits on the surface of the disk. This

TABLE II.—SURFACE PITTING TENDENCY OF LUBRICANTS

Lubricants with Which Pitting Occurs
Aluminum Stearate
Magnesium Stearate
Sodium Stearate
Stearic Acid
Stearyl Alcohol
Talc
Lubricants with Which Pitting Does Not Occur
Sodium Lauryl Sulfate
Sodium Oleate

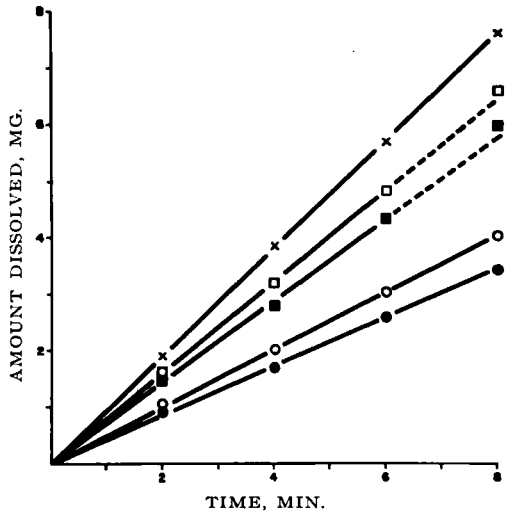

Fig. 8.—Effect of lubricants on initial dissolution rate of salicylic acid from rotating disks. Key: ●, 3% magnesium stearate; O, 1.5% magnesium stearate; ■, 3% aluminum stearate; □, 3% stearic acid; ×, no lubricant.

represents an increase in surface area which results in a more rapid dissolution of drug from the rotating disks. Although pits develop also on the surface of static disks, no increase in apparent dissolution rate occurs because the boundary layer at the solid-solvent interface of static disks is sufficiently thick to cover the pits (1). Pitting does not occur on the surface of disks which contain a water-soluble lubricant or no lubricant at all (Fig. 7). It does occur whenever any of the hydrophobic lubricants that were studied are used (Table II).

A comparative evaluation of the effect of various lubricants on dissolution rate by the rotating disk method had to be carried out in a manner which prevented complications because of pitting. This was accomplished by determining only initial dissolution rates, (*i.e.*, before apparent increases due to pitting had occurred). The results of the experiments are shown in Figs. 8 and 9. Magnesium stearate, aluminum stearate, sodium stearate, stearic acid, and talc all decreased the dissolution rate. Evaluation of relative effects of these agents is complicated because there is no assurance that their specific and/or effective surface areas were exactly equal, but magnesium stearate appears to have the most pronounced retarding effect. A concentration dependency is evident from the difference between the dissolution rate from disks containing 1.5% and 3%, respectively, of magnesium stearate. It is interesting that magnesium stearate not only retards dissolution rate more than does stearic acid, but that it also prolongs tablet disintegration time more than stearic acid (3).

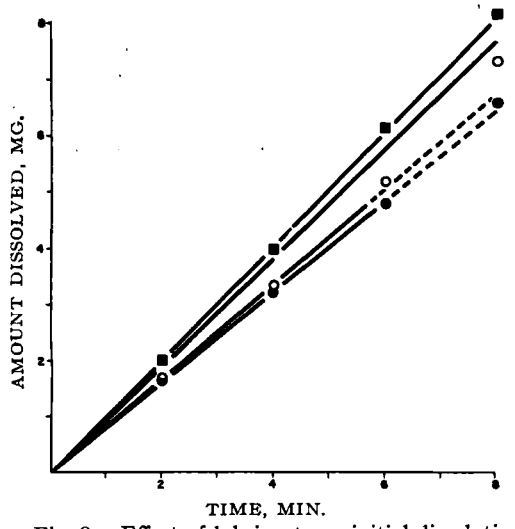

Fig. 9.—Effect of lubricants on initial dissolution rate of salicylic acid from rotating disks. Key: ●, 3% sodium stearate; O, 3% talc; ■, 3% sodium oleate; ——, no lubricant (from Fig. 8).

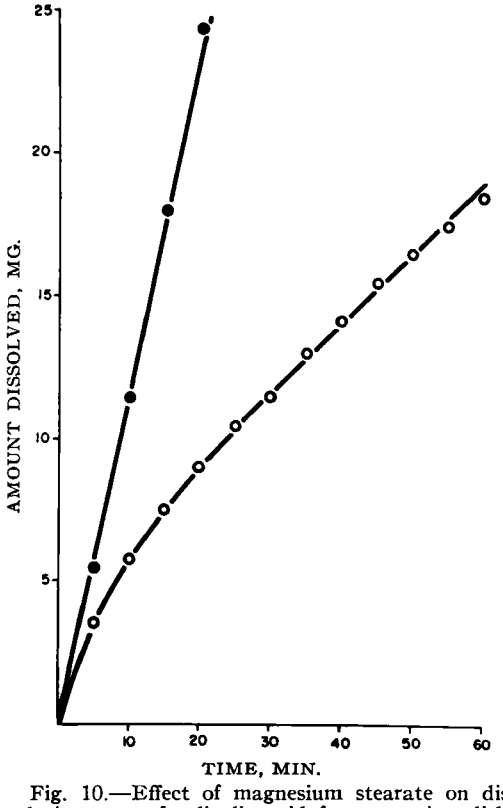

Fig. 10.—Effect of magnesium stearate on dissolution rate of salicylic acid from rotating disks made from fine salicylic acid powder. Key: O, 3% magnesium stearate; ●, no lubricant added.

The water-soluble sodium oleate had no significant effect on dissolution rate from disks and neither did sodium lauryl sulfate (Figs. 5 and 9). The dissolution rate retarding and pitting effect of sodium stearate appears unusual since this substance is stated to be water-soluble (13). However, it is also stated to be slowly dissolving (13). Addition of milligram amounts of sodium stearate to 0.1 *N* hydrochloric acid at 37° (the dissolution medium used in these studies) did not result in solution (and possible reprecipitation) of the substance, and it must be assumed that sodium stearate acts like a poorly soluble and hydrophobic agent under the conditions of this study.

One experiment was conducted with rotating disks

made from salicylic acid powder (100 mesh) and 3% magnesium stearate in order to determine whether a marked increase in the ratio of surface areas of drug:lubricant would result in a quantitative modification of the dissolution-retarding effect. The results of the experiment (shown in Fig. 10) indicate that the dissolution-retarding effect of magnesium stearate was somewhat greater in the case of disks prepared from salicylic acid powder (compared with disks made from granules). Therefore, the lubricant content of disks made from granules was sufficient to cover the surface of all granules; otherwise the retarding effect of lubricant would have decreased when drug granules were replaced by finely powdered drug. The initial curvature in the dissolution curve of magnesium stearate–containing disks made from salicylic acid powder (Fig. 10) was found repeatedly and may be because of a lower concentration of lubricant at the surface of the disk, from which it could have been removed mechanically by handling or during compression of the disk.

On the basis of these experiments it may be concluded that hydrophobic lubricating agents may retard the dissolution of drugs contained in compressed tablets not only by prolonging the disintegration time of both tablets and granules but also by reducing the area of the interface between drug particles and solvent. The surface of pellets made from salicylic acid and a hydrophobic lubricant represents a complex physicochemical system because the "true" surface area with respect to salicylic acid is difficult to estimate. This is the reason why the boundary layer thickness calculations in the first paper of this series were stated to be only reasonable approximations (1).

The dissolution rate enhancing effect of sodium lauryl sulfate appears to be due to increased wetting and to better solvent penetration into the tablets and granules as a result of the interfacial tension lowering effect of the surfactant. This conclusion is in accord with observations by Wurster and Seitz (12), who have found that dissolution of pellets with artificial pores is more rapid when the dissolution medium contains sodium lauryl sulfate, due mainly to better contact between solvent and the drug surface. Since sodium lauryl sulfate has excellent tablet lubricating activity (6), its use for this purpose appears advantageous, except where specific incompatibility or stability problems are present.

Of interest is a recent case where the addition of a surfactant to a tablet formulation resulted in better absorption of the drug (14). This was attributed first to a physiologic effect of the surfactant (an unlikely possibility because of the small amount of surfactant involved), but subsequent studies revealed that this was a dosage form effect (15, 16). It is apparent from the results of the present study that a surfactant can enhance the dissolution rate of drugs contained in compressed tablets and thereby cause them to be more rapidly and more completely absorbed.

CONCLUSIONS

It may be concluded from the results of this investigation that hydrophobic tablet lubricants can retard the dissolution of drugs from compressed tablets. The magnitude of this effect may be expected to depend, among other factors, upon the particular lubricant, its concentration, its particle size, the drug, the tablet formulation, various processing variables, and the conditions (particularly with respect to agitation intensity) under which dissolution is taking place. Hydrophilic lubricants apparently do not retard dissolution[3] and, if they have surface-tension lowering capability, may enhance the rate of dissolution of drugs contained in compressed tablets.

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[3] This statement is made with the qualification that surfactants can retard dissolution in certain systems because of their adsorption on the surface of the dissolving substance (17) or possibly by facilitating particle aggregation (18). However, it is felt that these effects would not be important with compressed tablets containing very small amounts of a surface active lubricant.

Mechanism of Sustained-Action Medication

Theoretical Analysis of Rate of Release of Solid Drugs Dispersed in Solid Matrices

By T. HIGUCHI

Theoretically expected rates of release of solid drugs incorporated into solid matrices have been derived for several model systems. Mathematical relations have been obtained for cases (a) where the drug particles are dispersed in a homogeneous, uniform matrix which acts as the diffusional medium and (b) where the drug particles are incorporated in an essentially granular matrix and released by the leaching action of the penetrating solvent. Release from both planar surface and a sphere is considered. The unidimensional release rates are shown to follow our earlier equation derived for release from ointment bases. Release rates from spherical pellets by both model mechanisms are shown not to followfi rst-order relationships. The analyses suggest that for the latter system the time required to release 50 per cent of the drug would normally be expected to be approximately 10 per cent of that required to dissolve the last trace of the solid drug phase in the center of the pellet.

THE PRESENT COMMUNICATION is concerned with the results of a theoretical analysis of mathematical relationships governing the rate of release of solid drugs randomly dispersed in solid matrices. Systems of this type have been widely studied and utilized recently as bases for dosage forms which provide more or less continuous release of medicaments over relatively long periods (1). Both Wiegand and Taylor (2) and Wagner (3) showed that per cent released time data reported in the literature for many sustained-release preparations give linear pseudo (or apparent) first-order rates over the terminal portions of the data from about 0.5 hour to the time the test was completed. The present study is an effort in relating the rate of release of drugs from such systems to the pertinent physical constants based on simple laws of diffusion.

Two geometric systems have been considered: (*a*) unidirectional leaching or extraction from a simple planar surface, and (*b*) three dimensional leaching or extraction from a spherical pellet. This would correspond most closely to the release process from an insoluble tablet matrix or certain sustained-action pellets.

Two mechanisms of release from these systems have been treated. (*a*) Extraction of the medicament by a simple diffusional process through and from an enveloping, homogeneous matrix. The drug is presumed to go successively from the crystal surfaces into the uniform matrix and out into the bathing solvent which in turn acts as a perfect sink. (*b*) Leaching of the medicament by the bathing fluid which is able to enter the drug-matrix phase through pores, cracks, and intergranular spaces. The drug is presumed to dissolve slowly into the permeating fluid phase and to diffuse from the system along the cracks and capillary channels filled with the extracting solvent. Intragranular diffusion is assumed, in this instance, to be insignificant. The two mechanisms are depicted schematically in Fig. 1.

It should be explicitly pointed out that the analyses reported here relate to these particular model systems, whereas the analyses of Wiegand and Taylor (2) and of Wagner (3) related to release data derived from formulated sustained-release or prolonged-action formulations. Actual dosage systems may be complicated by (*a*) simultaneous break-up of the matrix, (*b*) partial dissolution of the matrix substances, (*c*) one fraction of the dose being in a matrix form and the remainder of the dose being in a different, nonmatrix and readily available form, and (*d*) drug on the surface being released more rapidly than drug in the matrix. Where such complications are absent, the treatments under *Theoretical Analysis* are believed to yield the correct relationships.

THEORETICAL ANALYSIS

Release from a Planar System Having a Homogeneous Matrix.—The amount of total drug released from such a system into a bathing medium acting essentially as a perfect sink would be determined by the relationship

$$Q = \sqrt{Dt(2A - C_s)C_s} \qquad \text{(Eq. 1)}$$

where Q = the amount of drug released after time t per unit exposed area, D = the diffusitivity of the drug in the homogeneous matrix media, A = the total amount of drug present in the matrix per unit volume, and C_s = the solubility of the drug in the

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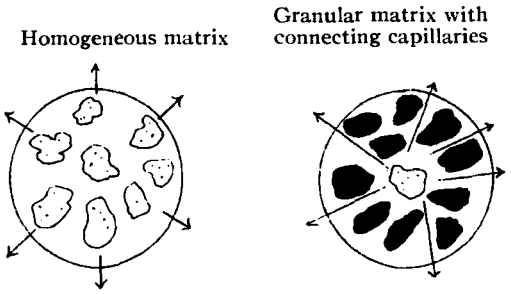

Fig. 1.—Two methods of drug release from the pellets.

matrix substance. We initially derived this equation for release from an ointment base containing finely dispersed drugs (4), but it is evident that it would be equally applicable for release from a sustained-action matrix of this type.

Release from a Planar System Having a Granular Matrix.—For the leaching type release mechanism occurring through diffusion movement utilizing intergranular openings, the above relation must be modified for the effective volume where diffusion can occur and the effective diffusional path. It can readily be seen for this system that

$$Q = \sqrt{\frac{D\epsilon}{\tau}(2A - \epsilon C_s)C_s t} \qquad \text{(Eq. 2)}$$

where Q = the amount of drug released after time t per unit exposed area, D = the diffusitivity of the drug in the permeating fluid, τ = the tortuosity factor of the capillary system $\geqq 3$, A = the total amount of drug present in the matrix per unit volume, C_s = the solubility of the drug in the permeating fluid, and ϵ = the porosity of the matrix.

The derivation of the above expression is essentially the same as Eq. 1, except that the cross-sectional area of the diffusional path must be reduced by the porosity factor ϵ, and the apparent solubility of the drug in the total system per unit volume must also be decreased by the same factor. The tortuosity factor, τ, is introduced to correct, in the same sense used in the classical Kozeny equation, for the lengthened diffusional path caused by the necessary lateral excursions.

For both equations the derivation (4) is based on the existence of a pseudo steady state condition during the release process and on the assumption that the drug particles are quite small relative to the average distance of diffusion and are uniformly distributed in the matrix. The equations would be essentially valid for systems in which A is greater than C_s or ϵC_s by a factor of three or four. Of course, if $A < C_s$ or ϵC_s, the drug would no longer be present as a solid and a different equation would apply.

Since the porosity factor in Eq. 2 refers, of course, to the porosity of the leached portion of the pellet, it differs from the initial porosity of the initially formed matrix. The difference would correspond directly to the volume of free space previously occupied by the extracted component or components. Thus

$$\epsilon = \epsilon_o + KA$$

for systems where the drug is the only extractable component, K being introduced to convert A to its corresponding volume fraction. K is equal to the specific volume of the drug = 1/(density of the drug) if A is expressed in terms of grams of drug per milliliter. For those instances where the initial porosity, ϵ_o, is very small or where the fraction of the matrix volume occupied by the drug is relatively large $\epsilon \cong KA$ and Eq. 2 reduces to $Q = A\sqrt{DK/\tau(2 - KC_s)C_s t}$. Thus for these systems it would appear that the fraction of the drug released at anytime is essentially independent of A.

Release from a Spherical Pellet Having a Homogeneous Matrix.—Any attempt to derive an exact solution for a system of this type is, of course, impossible since it would require an exact coordinate description of the distribution pattern of the dispersed particles. A reasonably accurate and useful mathematical solution can be based on the same assumption which permitted solution of the two-dimensional system. We can again assume for the case $A \gg C_s$ that a pseudo steady state condition would exist during the leaching or extraction process and that a sharp front will be formed between the partly leached or extracted part of the sphere and the untouched portion. This state of affairs is shown in Fig. 2 where a_o = the radius of the whole pellet, a' = the radius of that part still unextracted, and a = the polar radius of any region under consideration, and the remaining symbols are the same as before.

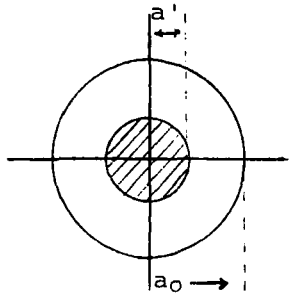

Fig. 2.—Schematic diagram of drug distribution in a partly extracted pellet.

It is evident in such a system that the concentration gradient is essentially zero for $a < a'$. The concentration in the region between a' and a_o will be a function of a and is assumed to be that fixed by Fick's first law.

Under pseudo steady state conditions as described above the total amount of material, SdQ, being released per unit time, dt, will be given by Fick's first law as

$$\frac{SdQ}{dt} = -4\pi a^2 D\frac{dC}{da} \qquad \text{(Eq. 3)}$$

where S is the diffusional area for $a' \leqq a \leqq a_o$. Integrating this from a to a_o we obtain

$$\left(\frac{SdQ}{dt}\right)_t\left(\frac{1}{a} - \frac{1}{a_o}\right) = 4\pi DC_a$$

where C_a = concentration at a or

$$\left(\frac{SdQ}{dt}\right)_t = \frac{4\pi DC_a}{\left(\frac{1}{a} - \frac{1}{a_o}\right)} = \frac{4\pi DC_s}{\left(\frac{1}{a'} - \frac{1}{a_o}\right)} \qquad \text{(Eq. 4)}$$

since $C_a = C_s$ at $a = a'$ and $C_a = 0$ at $a = a_o$ and

$$C_a = C_s \frac{a'}{a} \frac{(a_o - a)}{(a_o - a')} \quad \text{(Eq. 5)}$$

an expression which relates concentration to a in the region $a' < a < a_o$.

It is apparent that the total amount of the drug contained by the pellet at time = t is the sum of that in the unleached portion ($a < a'$) and that in the region no longer saturated with the drug ($a' < a < a_o$). Or the total residual amount of drug equals

$$\frac{4}{3}a'^3\pi A + \int_{a'}^{a_o} 4\pi a^2 C_a da \quad \text{(Eq. 6)}$$

$$= \frac{4}{3}\pi a'^3 A + \int_{a'}^{a_o} 4\pi a^2 C_s \frac{a'}{a}\frac{(a_o - a)}{(a_o - a')} da$$

$$= \frac{4}{3}\pi a'^3 A + \frac{4}{6}\pi C_s \frac{a'}{a_o - a'}(a_o^3 - 3a_o a'^2 + 2a'^3)$$

$$= 4\pi\left[\frac{a'^3}{3}A + \frac{C_s}{6}a'(a_o^2 + a'a_o - 2a'^2)\right] \quad \text{(Eq. 7)}$$

The change in the residual drug concentration corresponding to a change $-da'$ would then be

$$= 4\pi\left[Aa'^2 + \frac{C_s}{6}(a_o^2 + 2a'a_o - 6a'^2)\right](-da') \quad \text{(Eq. 8)}$$

This should in turn be equal but opposite in sign to the total flux over the period involved

$$\left(\frac{SdQ}{dt}\right)dt = \frac{4\pi DC_s}{\left(\frac{1}{a'} - \frac{1}{a_o}\right)}dt =$$

$$4\pi\left[Aa'^2 + \frac{C_s}{6}(a_o^2 + 2a'a_o - 6a'^2)\right]da'$$

$$DC_s dt = \left(\frac{a_o - a'}{a_o a'}\right)\left[Aa'^2 + \frac{C_s}{6}(a_o^2 + 2a'a_o - 6a'^2)\right]da' \quad \text{(Eq. 9)}$$

Integrating from a_o to a' we obtain

$$A(a_o^3 + 2a'^3 - 3a_o a'^2) +$$

$$C_s\left(4a'^2 a_o + a_o^3 \ln\frac{a_o}{a'} - a_o^3 - a_o^2 a' - 2a'^3\right) = 6DC_s a_o t \quad \text{(Eq. 10)}$$

For $C_s \ll A$ the above reduces to

$$a_o^3 - 3a'^2 a_o + 2a'^3 = \frac{6a_o}{A}DC_s t \quad \text{(Eq. 11)}$$

Equation 10 represents a general solution to the proposed problem since it permits determination of a' as a function of time, t, if the constants of the system, a_o, A, C_s, and D are known. Since the residual amount of drug at any time is already expressed by Eq. 7 in terms again of a', the total amount of drug released as a function of time can be readily calculated. Since any attempt to convert Eq. 10 into an explicit solution for a' as a function of t would result in a cumbersome relationship, it is more feasible in practice to obtain t as a function of a' and the amount of release also as a function of a' from Eq. 7 and then correlate the two dependent variables.

A general solution to Eq. 11 can be obtained in this implicit manner. For this it is more convenient to transform Eq. 11 into a dimensionless relationship

$$1 - 3\left(\frac{a'}{a_o}\right)^2 2\left(\frac{a'}{a_o}\right)^3 = \frac{6DC_s}{Aa_o^2}t = BT \quad \text{(Eq. 12)}$$

In this form the left hand expression in terms of a'/a_o is dimensionless and is independent of any units of measure employed. The factor, $B = 6DC_s/Aa_o^2$ is dimensionless except for time and can be calculated from the constants of the system. Since the fraction of drug remaining in the pellet for this system where $A \gg C_s$ would be

$$\text{Residual fraction of drug in pellet} = \left(\frac{a'}{a_o}\right)^3 \quad \text{(Eq. 13)}$$

we can readily prepare a plot of the residual fraction remaining in the pellet as a function of relative time. Actual time unit can be substituted in real systems where the constants comprising B are determinable. The results are shown for different (a'/a_o) values in Table I and are plotted in Fig. 3. It is evident

TABLE I.—CALCULATION OF RELEASE RATE FROM SPHERICAL PELLET $A \gg C_s$[a]

Time Scale		Drug Remaining (%) in Pellet
$\left(\frac{a'}{a_o}\right)$	Bt	$\left(\frac{a'}{a_o}\right)^3 \times 100$
1.00	0.0000	100.0
0.990	0.00030	97.03
0.980	0.001184	94.12
0.950	0.00725	85.70
0.90	0.02800	72.90
0.80	0.1040	51.20
0.70	0.2160	34.30
0.60	0.3520	21.60
0.50	0.5000	12.90
0.40	0.6480	6.40
0.30	0.7840	2.70
0.20	0.8960	0.800
0.10	0.9720	0.100
0.00	1.000	0.000

[a] Equation 11.

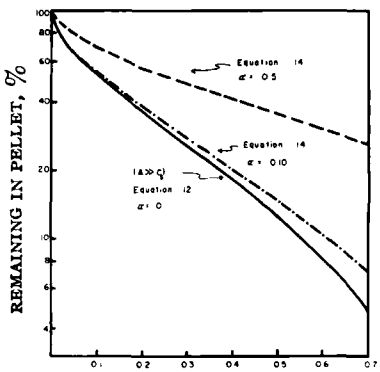

Fig. 3.—Influence of $\alpha = C_s/A$ values on rate of release.

from the table that the physical significance of B is that for the case $A \gg C_s$ its reciprocal corresponds to that time when the last trace of the solid drug dissolves into the matrix. Under these conditions the pellet can be considered to be totally exhausted. Also from the table it is apparent that the drug is very rapidly released at the beginning, approximately 50% of it being released at $t = 0.1 \times 1/B$.

The values shown in Table I have been plotted in Fig. 3 in the usual semilogarithmic fashion to show that the release rate predicted by this mechanism departs significantly from the first-order behavior. Aside from what appears to be a large initial surge of release, however, the plot yields a surprisingly linear relationship over a relatively wide range. This seems to arise from the fact that it is actually sigmoidal in nature and the apparent linear relationship observed (2, 3) may for some system be coincidental and for others be because of different overall mechanism.

In Fig. 4 the fraction of drug released from a sphere calculated from Eq. 11 is compared to that expected from Eq. 1 on the basis that the total exposed surface is $4\pi a_o^2$. The plot has been made as a function of $(Bt)^{1/2}$ since this affords a linear relationship for the two-dimensional system. It is evident that at the beginning the two equations predict a similar extent of release, as expected, since a plane of the same area would be a good approximation for the sphere in this phase. It is only beyond 50% release that significant deviation is evident between the two equations.

The more general Eq. 10 which takes into account the amount of drug remaining in the partly extracted region can be solved in an identical manner based on Eqs. 7 and 10. Setting $C_s = \alpha A$ and rearranging Eq. 10 we obtain

$$1 + 2\left(\frac{a'}{a_o}\right)^3 - 3\left(\frac{a'}{a_o}\right)^2 + \alpha\left[4\left(\frac{a'}{a_o}\right)^2 + ln\,\frac{a_o}{a'} - \frac{a'}{a_o} - 2\left(\frac{a'}{a_o}\right)^3 - 1\right] = \frac{6DC_st}{Aa_o^2}$$

or

$$1 - \alpha + 2(1-\alpha)\left(\frac{a'}{a_o}\right)^3 - (3 - 4\alpha)\left(\frac{a'}{a_o}\right)^2 - \alpha\left(\frac{a'}{a_o}\right) + \alpha\, ln\,\frac{a_o}{a'} = \frac{6DC_st}{Aa_o^2} \quad \text{(Eq. 14)}$$

Release behavior predicted by this equation for several values of α are also shown in Fig. 3. Residual fraction in these instances based on Eq. 7 would be

$$\text{Residual Fraction} = \left(\frac{a'}{a_o}\right)^3 + \frac{\alpha}{2}\left[\left(\frac{a'}{a_o}\right) + \left(\frac{a'}{a_o}\right)^2 - 2\left(\frac{a'}{a_o}\right)^3\right] \quad \text{(Eq. 15)}$$

Since in most real systems α is usually very small, any term containing it as a factor can normally be ignored.

Plots shown in Fig. 3 are, in a sense, universal relationships in that they permit estimation of the release behavior for all systems for given α values. They indicate that the relative release rates (*i.e.*, the fraction released per unit time) from pellets of this type would be inversely proportional to the square of the radius of the pellet and the drug concentration but directly proportional to the solubility and the diffusitivity of the drug. The plot shown for $\alpha = 0.5$ is less valid than the others since the assumed pseudo steady state condition requires A to be significantly larger than C_s. Actually α must be considerably smaller for the spherical three-dimensional system for the steady state situation to exist than for the planar system. This is especially true for higher states of depletion.

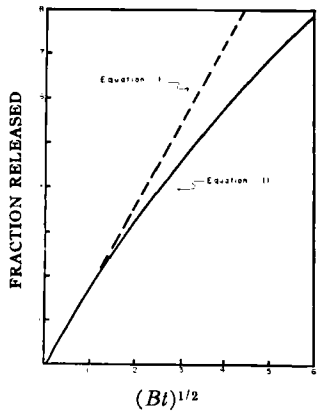

Fig. 4.—Comparison of Eqs. 1 and 11.

Release by Leaching from Granular Spherical Pellet.—The solution to the rate of leaching by external solvent (*e.g.*, gastric fluid) of solid drugs uniformly dispersed in granular spherical pellets can be developed exactly as in the preceeding case. It is evident that the dimensionless expression corresponding to Eq. 14 will take the form

$$(1-\alpha) + 2\left(\frac{a'}{a_o}\right)^3(1-\alpha) - \left(\frac{a'}{a_o}\right)^2(3 - 4\alpha) - \alpha\left(\frac{a'}{a_o}\right) + \alpha\, ln\left(\frac{a_o}{a'}\right) = \frac{6D\epsilon C_st}{\tau Aa_o^2} \quad \text{(Eq. 16)}$$

where $\alpha = \epsilon C_s/A$ and $\epsilon = \epsilon_o + KA$, and the remaining symbols the same meanings as before with D being the diffusitivity in the solvent. If as in the two-dimensional case we take the initial porosity, ϵ_o, as being negligible we obtain

$$(1-\alpha) + 2\left(\frac{a'}{a_o}\right)^3(1-\alpha) - \left(\frac{a'}{a_o}\right)^2(3 - 4\alpha) - \alpha\left(\frac{a'}{a_o}\right) + \alpha\, ln\left(\frac{a_o}{a'}\right) = \frac{6DKC_st}{\tau a_o^2}$$

For $\alpha \ll 1$, this reduces to

$$1 + 2\left(\frac{a'}{a_o}\right)^3 - 3\left(\frac{a'}{a_o}\right)^2 + \frac{6DKC_st}{\tau a_o^2} \quad \text{(Eq. 17)}$$

where the residual fraction $= (a'/a_o)^3$.

In these analyses it has been tacitly assumed that the time of extraction begins with the slightly porous pellet already permeated by the extracting solution. Since in practice such pellets (or "cores") will be taken dry there will be a short lag time corresponding to that required to wet the interior of the matrix. This time, however, should normally be relatively small compared to the duration of action of such medications.

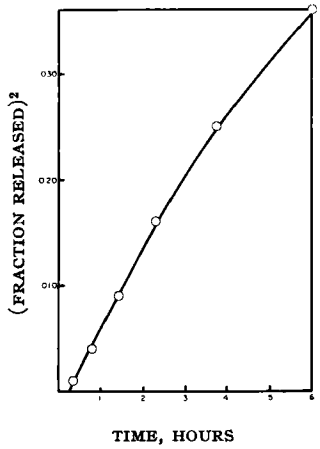

Fig. 5.—Comparison of Simoons' experimental data (5) with Eq. 17.

GENERAL DISCUSSION

The several solutions to the release behavior of the systems considered are believed to be essentially exact for the models employed. Experimental data available appear to substantiate these analyses (5).

In real systems, however, a number of other factors may come into play which may modify the total behavior. The models employed assume that the systems are neither surface coated, nor that their matrices undergo significant alteration in the presence of moisture. Since in real systems these play varying roles in modifying the release pattern of sustained-action dosage forms, any attempt to apply these equations must be made with this in mind.

Other serious deviations from the derived relationship may occur for systems which tend to differ significantly from the adopted models. For example, for pellets containing a relatively high percentage of drugs the leaching process would tend to weaken the matrix structure and produce erosion. This may play a significant role in altering the observed real rate. Another effect which is not considered in these treatments is the influence of solvent flow induced within the pellets by external agitation. This effect, however, will be important only with pellets of relatively high porosity.

In Fig. 5 data reported by Simoons in his interesting paper on experimental measurements of release rate of sustained-action medication (6) for relatively insoluble drug hard compressed (his Fig. 15) are plotted in the form of square of fraction released against extraction time. The extraction data show a short lag time probably corresponding to that required to wet the pellets. This is followed by a release pattern closely matching that predicted by Eq. 17, the smooth line representing the theoretical values and the points, the experimentally observed values reported by Simoons. The total theoretical curve was based on experimentally observed time for 50% release and the initial lag time.

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Dielectric Constants of Complex Pharmaceutical Solvent Systems I

Water-Ethanol-Glycerin and Water-Ethanol-Propylene Glycol

By D. L. SORBY, R. G. BITTER, and J. G. WEBB

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MOORE (1) HAS PRESENTED a method wherein manipulation of solvent dielectric constant is utilized to produce dissolution of a solute at a desired concentration and to blend pharmaceutical solvents to a predetermined degree of polarity. In this method, certain simplifying assumptions are made, one being that the dielectric constant of a complex solvent mixture may be calculated to a good approximation by taking the sum of the products of volume composition and dielectric constant for each individual component in the mixture. This method of calculating dielectric constants of complex mixtures is based on a simplification of the Onsager-Kirkwood

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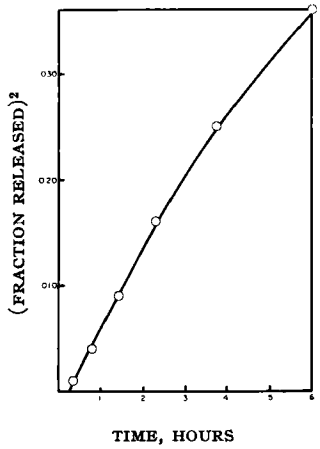

Fig. 5.—Comparison of Simoons' experimental data (5) with Eq. 17.

GENERAL DISCUSSION

The several solutions to the release behavior of the systems considered are believed to be essentially exact for the models employed. Experimental data available appear to substantiate these analyses (5).

In real systems, however, a number of other factors may come into play which may modify the total behavior. The models employed assume that the systems are neither surface coated, nor that their matrices undergo significant alteration in the presence of moisture. Since in real systems these play varying roles in modifying the release pattern of sustained-action dosage forms, any attempt to apply these equations must be made with this in mind.

Other serious deviations from the derived relationship may occur for systems which tend to differ significantly from the adopted models. For example, for pellets containing a relatively high percentage of drugs the leaching process would tend to weaken the matrix structure and produce erosion. This may play a significant role in altering the observed real rate. Another effect which is not considered in these treatments is the influence of solvent flow induced within the pellets by external agitation. This effect, however, will be important only with pellets of relatively high porosity.

In Fig. 5 data reported by Simoons in his interesting paper on experimental measurements of release rate of sustained-action medication (6) for relatively insoluble drug hard compressed (his Fig. 15) are plotted in the form of square of fraction released against extraction time. The extraction data show a short lag time probably corresponding to that required to wet the pellets. This is followed by a release pattern closely matching that predicted by Eq. 17, the smooth line representing the theoretical values and the points, the experimentally observed values reported by Simoons. The total theoretical curve was based on experimentally observed time for 50% release and the initial lag time.

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MOORE (1) HAS PRESENTED a method wherein manipulation of solvent dielectric constant is utilized to produce dissolution of a solute at a desired concentration and to blend pharmaceutical solvents to a predetermined degree of polarity. In this method, certain simplifying assumptions are made, one being that the dielectric constant of a complex solvent mixture may be calculated to a good approximation by taking the sum of the products of volume composition and dielectric constant for each individual component in the mixture. This method of calculating dielectric constants of complex mixtures is based on a simplification of the Onsager-Kirkwood

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equation and would theoretically be correct only if the mixture of substances exhibited properties of an ideal solution. Since most common pharmaceutical solvent systems are composed of substances which exhibit a high degree of intermolecular association and are seldom ideal in their behavior, dielectric constants of such systems would not usually be expected to be a simple additive function of the concentrations of the components as described above. In these situations, the method of obtaining the dielectric constant by calculation would be expected to give only rough approximations of the true value for the solvent mixture in question. It was also stated (1) that while more precise work would require that compositions of the solvent mixtures be stated on the basis of weight per cent, volume compositions could be substituted in the calculations for convenience in formulation work. It was implied that as a result of expressing concentration on a volume percentage basis, the calculated values would not be as precise as when weight per cent had been used.

Decroocq and Jungers (2), however, have presented data showing that if concentrations of the individual components are expressed on the basis of volume percentage when using the simplified form of the Onsager-Kirkwood equation described above, agreement between experimental and calculated dielectric constants is better than when concentrations are expressed on the basis of mole fraction. From results of studies on binary mixtures exhibiting ideal or nearly ideal behavior, Decroocq and Jungers estimated that calculated values would usually be within 1% of experimental values when volume percentage is used as the concentration variable. Their report also presented data showing large deviations between experimental and calculated dielectric constants when the binary solutions studied were non-ideal in their behavior.

Thus one might expect that use of volume per-

TABLE I.—SUMMARY OF DATA OBTAINED FOR TEST SAMPLES IN THE WATER-ETHANOL-GLYCERIN SYSTEM AT 25°C.[a]

Ratio of Glycerin to Ethanol[c]	Water Content of Test Sample[b] 20% w/w	40% w/w	60% w/w	80% w/w	Other
1:0	52.4 (1.2107)	62.6 (1.1594)	68.9 (1.1024)	74.2 (1.0487)	[d]
	52.2	62.5	70.0	75.1	
4:1	45.7 (1.1266)	57.0 (1.0971)	67.3 (1.0656)	74.0 (1.0325)	[e]
	45.6	55.9	67.7	73.7	
7:3	44.4 (1.0814)	54.9 (1.0678)	64.8 (1.0479)	72.3 (1.0244)	[f]
	44.1	55.3	64.8	72.1	
1:1	38.4 (1.0027)	50.1 (1.0166)	61.6 (1.0135)	71.5 (1.0062)	
	38.5	50.1	61.7	71.1	
3:7	36.3 (0.9330)	46.0 (0.9600)	58.9 (0.9812)	70.3 (0.9917)	
	36.2	46.5	59.1	70.5	
1:4	34.5 (0.9003)	44.2 (0.9366)	57.3 (0.9640)	69.3 (0.9832)	
	34.4	44.4	57.0	69.7	
0:1	31.7 (0.8423)	54.2 (0.9351)	41.2 (0.8900)	68.1 (0.9704)	[g]
	31.6	54.0	41.4	68.1	

[a] The first figures in each section are dielectric constant values measured for the duplicate samples. The figure in parenthesis is the specific gravity of one test sample. [b] The amount of water contained in the commercial products of ethanol and glycerin was included in calculation of the water content of test samples. [c] These ratios represent the ratio of 100% glycerin to 100% ethanol. Water contained in the commercial products of ethanol and glycerin was accounted for in preparing all samples. [d] 5.0% w/w—43.0, 43.0. [e] 5.0% w/w—38.2, 38.2 (1.1397); 10% w/w—40.8, 40.7 (1.1382). [f] 10.0% w/w—38.7, 38.4 (1.0847); 15% w/w—41.2, 41.2 (1.0855). [g] 7.7% w/w 24.9, 25.2 (0.8107); 50.0% w/w—46.8, 47.0 (0 9132).

TABLE II.—SUMMARY OF DATA OBTAINED FOR TEST SAMPLES IN THE WATER-ETHANOL-PROPYLENE GLYCOL SYSTEM AT 25°C.[a]

Ratio of Propylene Glycol to Ethanol[c]	Water Content of Test Sample[b] 20% w/w	40% w/w	60% w/w	80% w/w	Other
1:0	38.6 (1.0742)	49.5 (1.0417)	62.4 (1.0313)	71.0 (1.0155)	
	40.1	51.5	63.2	72.0	
4:1	37.3 (0.9976)	48.6 (1.0089)	61.0 (1.0115)	70.9 (1.0049)	
	38.0	48.6	60.8	71.2	
3:2	36.2 (0.9548)	46.6 (0.9791)	59.4 (0.9918)	70.7 (0.9935)	[d]
	36.1	46.7	60.0	71.0	
2:3	33.7 (0.9135)	42.6 (0.9475)	57.3 (0.9720)	69.6 (0.9843)	[e]
	34.6	45.0	58.1	70.0	
1:4	32.5 (0.8777)	41.1 (0.9180)	54.2 (0.9532)	68.4 (0.9724)	[f]
	33.3	43.6	57.3	70.3	
0:1	31.7 (0.8423)	54.2 (0.9351)	41.2 (0.8900)	68.1 (0.9704)	[g]
	31.6	54.0	41.4	68.1	

[a] The first figures in each section are dielectric constant values measured for the duplicate samples. The figure in parenthesis is the specific gravity of one test sample. [b] The amount of water contained in the commercial samples of ethanol and propylene glycol was included in calculation of the water content of test samples. [c] These ratios represent the ratio of 100% propylene glycol to 100% ethanol. Water contained in commercial products was accounted for in preparing all samples. [d] 3.1% w/w—26.0, 25.8 (0.9302); 10% w/w—30.7, 30.7 (0.9431). [e] 5.0% w/w—25.9, 25.9 (0.8886); 12% w/w—30.0, 30.1 (0.9014). [f] 10% w/w—27.8, 27.7 (0.8566); 15% w/w—30.5, 30.5 (0.8649). [g] 7.7% w/w—24.9, 25.2 (0.8107); 50% w/w—46.8, 47.0 (0.9132).

centage in these calculations should not produce less precise results than when alternative methods, such as weight per cent or mole fraction, are used for expressing concentrations of the various components of a system.

In actual practice, precise values of dielectric constants of complex, multi-component solutions can best be obtained by direct experimental measurement. The scientific literature contains much data pertaining to dielectric constants of binary mixtures, *e. g.*, (2–5), but little information concerning the more complex mixtures utilized in pharmaceutical formulations. The objectives of this research were to (*a*) determine relationships existing between dielectric constant and composition experimentally in two common pharmaceutical solvent systems, water-ethanol-glycerin and water-ethanol-propylene glycol, (*b*) determine the accuracy of calculated dielectric constants and the method of expressing compositions (weight percentage, or volume percentage) producing the better agreement between experimental and calculated values for these complex systems. Information of this type would be useful for precise adjustment of solvent polarity in pharmaceutical formulation work and, in addition, would be of potential interest to various types of physicochemical investigations of the behavior of these complex non-ideal systems.

EXPERIMENTAL

Materials and Reagents.—Since data were desired for systems actually encountered in pharmaceutical formulation practice, the materials used in this study were of commercially available grades. The following materials were used without further purification; ethanol, 92.3% w/w; propylene glycol, 99.5% w/w; and glycerin, 95.0% w/w. Distilled water was used in preparing all samples.

Acetone, reagent grade, was distilled over calcium chloride and stored, protected from atmospheric moisture, until needed for calibrating the instrument used to measure dielectric constants of the test mixtures.

Preparation of Test Samples and Collection of Data.—Sets of test mixtures were prepared so that in a given set all samples contained the same ratio of ethanol to glycerin or ethanol to propylene glycol. The water content of mixtures in each set was varied throughout a range of concentration, usually including samples containing 20, 40, 60, and 80% by weight of water. The amount of water present in the stock solutions of ethanol, propylene glycol, and glycerin was taken into consideration in all calculations of solvent composition. Duplicates were prepared of each different sample.

The required amount of each ingredient needed to make a 100-Gm. quantity of sample was weighed with an accuracy of ±0.05 Gm. into glass-stoppered Pyrex bottles. The bottles were then immersed in a constant temperature bath at 25.0°. When thermal equilibrium was established, the specific gravity of each sample was determined by the pycnometer method using water at 25.0° as the reference solution. The remainder of the sample was returned in the thermostat until measurement of the dielectric constant was performed.

Dielectric constants of the test mixtures were determined with a Sargent, model V, chemical oscillometer standardized with water-acetone mixtures of known dielectric constant (6). Approximately 10 ml. of the test sample was pipetted into

Table III.—Weight Compositions of the Water-Ethanol-Glycerin System which are Expected to Give Identical Dielectric Constant Values

Dielectric Constant Value	Water, % w/w	Ethanol, % w/w	Glycerin, % w/w
70	61.7	0	38.3
	67.8	6.4	25.8
	73.5	8.0	18.5
	76.0	12.0	12.0
	79.3	14.5	6.2
	80.6	15.5	3.9
	82.8	17.2	0
60	34.7	0	65.3
	45.4	10.9	43.7
	49.8	15.1	35.1
	56.2	21.9	21.9
	61.8	26.7	11.5
	64.3	28.6	7.1
	68.4	31.6	0
50	16.5	0	83.5
	28.6	14.3	57.1
	30.5	20.8	48.7
	39.6	30.2	30.2
	45.7	38.0	16.3
	48.8	41.0	10.2
	54.3	45.7	0
40	8.7	18.3	73.0
	12.8	26.2	61.0
	20.4	39.8	39.8
	29.5	49.4	21.1
	33.3	53.4	13.3
	37.5	62.5	0

Table IV.—Weight Compositions of the Water-Ethanol-Propylene Glycol System which are Expected to Give Identical Dielectric Constant Values

Dielectric Constant Value	Water, % w/w	Ethanol, % w/w	Propylene Glycol, % w/w
70	74.8	0	25.2
	77.7	4.5	17.8
	78.7	8.5	12.8
	79.0	12.6	8.4
	81.5	14.8	3.7
	82.8	17.2	0
60	55.8	0	44.2
	58.5	8.3	33.2
	60.5	15.8	23.7
	61.5	23.1	15.4
	66.4	26.9	6.7
	68.4	31.6	0
50	40.0	0	60.0
	41.5	11.7	46.8
	45.3	21.9	32.8
	47.5	31.5	21.0
	51.7	38.6	9.7
	54.3	45.7	0
40	21.5	0	78.5
	24.5	15.1	60.4
	28.2	28.7	43.1
	32.5	40.5	27.0
	35.2	51.8	13.0
	37.5	62.5	0
30	9.3	36.3	54.4
	12.0	52.8	35.2
	14.3	68.6	17.1
	17.0	83.0	0

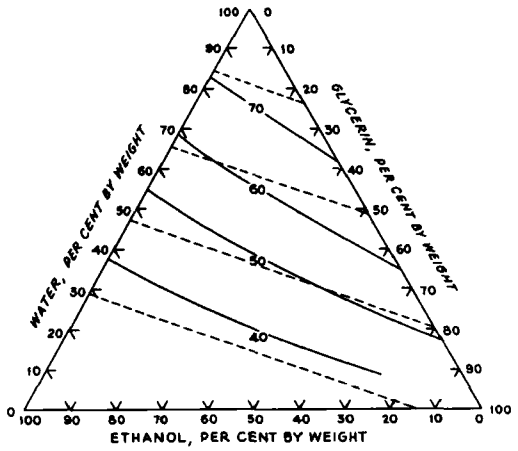

Fig. 1.—Dielectric constant-weight percentage relationships for the system water-ethanol-glycerin. Key: ————, experimental; - - - - - -, calculated.

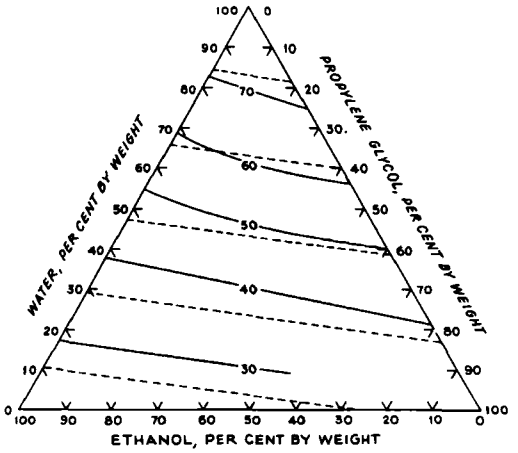

Fig. 2.—Dielectric constant-weight percentage relationships for the system water-ethanol-propylene glycol. Key: ————, experimental; - - - - - -, calculated.

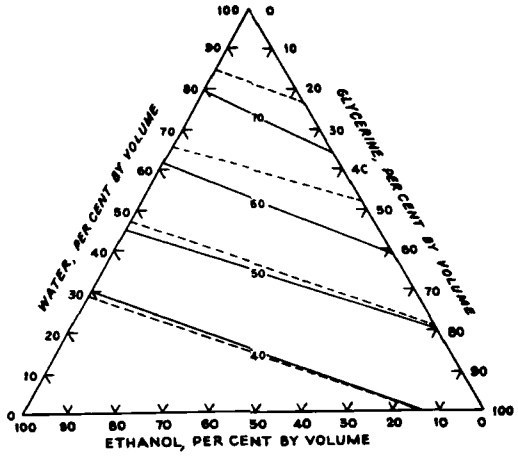

Fig. 3.—Dielectric constant-volume percentage relationships for the system water-ethanol-glycerin. Key: ————, experimental; - - - - - -, calculated.

the test cell of the oscillometer and the scale reading recorded. This measurement was made in duplicate for each sample, the test cell being cleaned and dried between each reading. The dielectric constant for each test mixture was obtained by comparing the scale reading on the oscillometer to a standard curve constructed by plotting dielectric constant against scale reading for the water-acetone standards described above.

Specific gravity and dielectric constant values determined for the various samples in the two systems of solvents tested are given in Tables I and II.

Treatment of Data.—Plots of dielectric constant *versus* weight per cent water were made for each set of samples having the same constant ratio of ethanol to glycerin or ethanol to propylene glycol. Water concentrations which theoretically would have been needed to produce samples having dielectric constant values of 70, 60, 50, etc., in each set were determined from curves drawn through points representing the experimental data. The amount of ethanol and glycerin or propylene glycol in each hypothetical sample was determined from a knowledge of their ratio in the sample and the difference between the per cent water and 100%. These derived solvent compositions (Tables III and IV) were plotted on a ternary diagram and tie lines were drawn which best fitted all points representing solutions expected to exhibit the same dielectric constant (Figs. 1 and 2). The per cent by volume compositions of the test samples were calculated by the usual methods from knowledge of their weight per cent compositions and experimentally determined specific gravities. Solutions theoretically giving dielectric constants of 70, 60, 50, etc., were determined graphically and plotted on ternary diagrams as described above (Figs. 3 and 4). The values derived from the experimental data and used to prepare Figs. 3 and 4 are summarized in Tables V and VI.

Dielectric constants measured for solutions of compositions given by points picked at random from the curves in Figs. 1–4 verified the accuracy of these curves as being within ±0.5 units of the dielectric constant specified for the given curve.

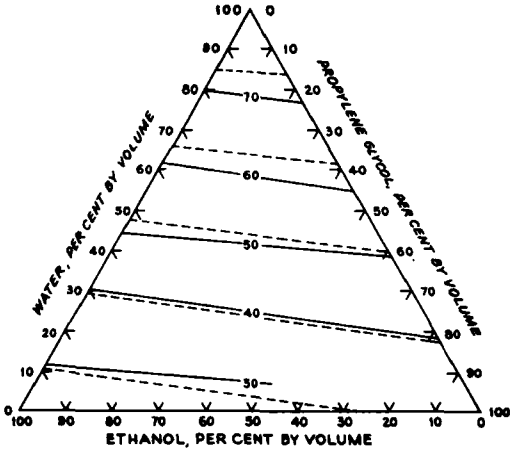

Fig. 4.—Dielectric constant-volume percentage relationships for the system water-ethanol-propylene glycol. Key: ————, experimental; - - - - - -, calculated.

TABLE V.—VOLUME COMPOSITIONS OF THE WATER-ETHANOL-GLYCERIN SYSTEM WHICH ARE EXPECTED TO GIVE IDENTICAL DIELECTRIC CONSTANT VALUES

Dielectric Constant Value	Water, % v/v	Ethanol, % v/v	Glycerin, % v/v
70	66.2	0	33.8
	68.5	9.0	22.5
	74.7	10.3	15.0
	75.6	15.0	9.4
	76.5	18.5	5.0
	77.8	19.2	3.0
	79.5	20.5	0
60	38.7	0	61.3
	47.5	15.0	37.5
	48.9	20.8	30.3
	54.8	27.8	17.4
	57.5	33.5	9.0
	59.6	35.0	5.4
	61.6	38.4	0
50	18.8	0	81.2
	28.0	20.5	51.5
	29.5	28.7	41.8
	37.1	38.7	24.2
	41.2	46.4	12.4
	43.2	49.2	7.6
	47.0	53.0	0
40	7.7	26.3	66.0
	11.3	36.1	52.6
	19.6	49.5	30.9
	24.6	59.5	15.9
	26.7	63.5	9.8
	30.2	69.8	0

TABLE VI.—VOLUME COMPOSITIONS OF THE WATER-ETHANOL-PROPYLENE GLYCOL SYSTEM WHICH ARE EXPECTED TO GIVE IDENTICAL DIELECTRIC CONSTANT VALUES

Dielectric Constant Value	Water, % v/v	Ethanol, % v/v	Propylene Glycol, % v/v
70	77.0	0	23.0
	77.0	5.7	17.3
	76.5	11.0	12.5
	76.6	15.5	7.9
	77.9	18.6	3.5
	79.5	20.5	0
60	55.0	0	45.0
	56.8	10.7	32.5
	57.6	19.8	22.6
	59.8	26.7	13.5
	60.0	33.6	6.4
	61.6	38.4	0
50	38.8	0	61.2
	40.2	14.8	45.0
	41.2	27.5	31.3
	43.5	37.5	19.0
	42.4	48.4	9.2
	47.0	53.0	0
40	18.8	0	81.2
	23.3	19.0	57.7
	24.3	35.4	40.3
	25.7	49.3	25.0
	25.8	62.3	11.9
	30.2	69.8	0
30	6.8	43.6	49.6
	8.8	60.5	30.7
	10.7	75.0	14.3
	11.8	88.2	0

DISCUSSION

In Figs. 1 and 2, dielectric constants have been plotted as a function of both calculated and experimentally determined solvent composition. It is apparent that rather large deviations exist between theoretical and experimental values. In addition, dielectric constant is not in most cases in these systems a linear function of the weight composition. These deviations are not unexpected when one considers the strong tendencies for interaction between the molecular species involved. While data for other ternary solvent systems are lacking in the scientific literature, deviations of this magnitude are found when calculated values of dielectric constants are compared with data of other investigators (3, 5) for water-ethanol and water-glycerin systems. Values of dielectric constants reported in the literature (3, 5) are in general good agreement with the values reported here, considering differences in methodology and sample purity.

Results shown in Figs. 3 and 4 indicate that curves relating dielectric constant and volume composition of samples are linear, although agreement between experimental and calculated values is poor, except in the solutions where dielectric constants equal 40 and 50. Moore (1) noted that deviations of dielectric constant from the direct proportionality predicted by mathematical computation should be more pronounced as polarity of the mixtures increases. Such is indeed the case in the two systems studied. The nature of the curves does indicate that dielectric constants in these systems are apparently some type of linear function of the concentration of the various components expressed on a volume basis; however, because of the various complexities of these systems, no simple relationship appears to exist between dielectric constants of the mixtures and those of the pure components which would allow computation by the simplified form of the Onsager-Kirkwood equation. There seems to be no major advantage to expressing concentrations on a weight basis; in fact, use of volume percentage appears to be of some benefit in regard to ease of fitting curves through the experimental points.

It has been our experience that the dielectric constant principle of blending solvent systems as outlined by Moore (1) sometimes fails to produce the desired results when used in actual practice. It is quite possible that some of these failures have been because of the large differences which can occur between calculated and actual dielectric constant values in these complex systems. We hope that the dielectric constant–composition relationships obtained as a result of our investigation can serve to place this useful technique of solvent blending on a more reliable basis. Experimental determinations of dielectric constants of other pharmaceutical systems and other important aspects of the relationships between solubility and dielectric constants are being continued and will be reported at a later date.

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Preliminary Identification of the Antibacterial Principle "Madronin" from the Leaves of *Arbutus menziesii*

By BALACHANDRA KABADI and E. ROY HAMMARLUND

The antibacterially active compound "madronin" from the leaves of Pacific Madrona tree has been tentatively identified as a mixture of gallotannin and catechol tannin in addition to varying amounts of their degradation products, gallic acid, m-digallic acid, possibly trigallic acid, catechol, phloroglucinol, and glucose. Experimental evidence includes results of qualitative physical and chemical analyses, light absorption, paper chromatography, and microbiological fermentation tests. A simple modified laboratory extraction and purification procedure for the active principle is given. The previous observations made on madronin are explained according to its newly determined composition. Since tannin has been shown to be a rather potent antibacterial substance for many micro-organisms, its possible influence upon the various surveys for antibacterial agents in higher plants should be considered.

In 1952 HAMMARLUND, Pennington, and Rising (1) reported that the leaves of the Pacific Madrona tree (*Arbutus menziesii* Pursh., family *Ericaceae*) contained one or more antibacterial substances which were active *in vitro* mainly against Gram-positive and acid-fast bacteria. Their studies indicated the presence of several substances having similar chemical and antibacterial activities and the name "madronin" was given to the crude mixture of the active material (1, 2).

This current investigation was undertaken to characterize further and identify chemically the antibacterial principle by employing various improvements in the extraction and purification procedures.

Since light absorption studies made in this current investigation and most of the earlier qualitative tests indicated that madronin was probably a tannin although a few chemical tests reported earlier gave doubtful reactions for the presence of phenolic groups (1), numerous comparison tests were conducted with such substances as tannic acid N.F. (from *Quercus infectoria*), gallotannin (from *Rhus senialita*), and polyhydroxy phenolic compounds.

The active antibacterial substance prepared by using the present modified extraction and purification procedure, which is similar to that routinely used (3) for the preparation of tannins, is referred to in this presentation as "purified" madronin.

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Abstracted from a thesis submitted by Balachandra Kabadi to the Graduate School, University of Washington, Seattle, in partial fulfillment of Master of Science degree requirements.

EXPERIMENTAL METHODS AND RESULTS

Modified Extraction Procedure.—Fresh leaves and stems were dried in a circulating hot air oven at 45° for about 48 hours. Six-hundred grams of the dried leaves were powdered and were repeatedly extracted in a Waring Blendor with 5 L. of distilled water. The aqueous extract was centrifuged, filtered, and dried under reduced pressure. The dried aqueous extract, weighing about 44 Gm., was repeatedly extracted in a large centrifuge tube by stirring with acetone in which sugar and other nonactive constituents were insoluble. The clear acetone solution was decanted following centrifugation and evaporated to dryness under reduced pressure. This yielded approximately 6.5 Gm. of material.

The residue from the acetone extract was dissolved in a minimal amount of water and filtered. This aqueous solution (approximately pH 3.7) was repeatedly extracted with ethyl ether to remove the coloring matter and gallic acid. To the aqueous solution 0.33 *M* phosphate buffer was added to raise the pH to 6.8, and the solution was extracted in several portions with 1 L. of ethyl acetate. The combined ethyl acetate solutions were air-dried and yielded about 4 Gm. of dried, antibacterially active residue.

Finally, the dried residue was dissolved in a minimal amount of water (approximately 15 ml.) and added to a 1 in. by 2-ft. chromatographic adsorption column packed with standard cellulose powder[1] prepared from a slurry in *n*-butanol (4). The chromatographic column was developed by the further addition of *n*-butanol, whereupon two colored bands formed. The lower band was eluted with *n*-butanol; the effluent was evaporated under reduced pressure and gave about 3.5 Gm. of yellow residue. The top purple band, which did not move with the butanol solvent, was eluted with water and rejected because it was antibacterially inactive.

The dried residue from the butanol effluent was dissolved in a minimal amount of acetone and poured slowly into 500 ml. of ethyl ether. A resultant white precipitate was filtered off and then immediately dried under reduced pressure. This was designated as "purified" madronin.

[1] Whatman B quality, standard grade, W. & H. Balton, Ltd., England.

TABLE I.—ANTIBACTERIAL ACTION OF MADRONIN AND RELATED SUBSTANCES

Test Compd.	Minimal Inhibition Concn., mcg. per ml. of the Medium
Madronin	4.5 to 5.0
"Purified" madronin	3.3 to 4.3
Tannic acid N.F.	2.5
Gallotannin[a]	4.9
Gallic acid[b]	11.6

[a] Gallotannin extracted from Chinese galls using 85% ethanol and dried under reduced pressure. [b] Mallinckrodt Chemical Works, New York, N. Y.

Antibacterial Study.—Tests made for the determination of the antibacterial activity of "purified" madronin and related substances were similar to those of Hammarlund, *et al.* (1). These tests were a quantitative serial dilution method for determining the minimal inhibitory concentration and a qualitative filter paper disk diffusion agar plate method for the qualitative detection of the antibacterial substances. The test organism was *Staphylococcus albus* C_{87},[2] and the media used for the serial dilution tests and agar plates was identical with that previously reported (1). The antibacterial activity of madronin, "purified" madronin, and related substances is recorded in Table I.

Qualitative Analyses of Antibacterial Principle.—The following chemical reactions which are generally characteristic of tannins were given by both the aqueous madronin and "purified" madronin solutions. Madronin formed a precipitate with gelatin, albumin, or starch (5). Madronin and tannic acid N.F. exhibited identical tanning properties with the Goldbeater's skin test using fresh untanned cowhide (3). Madronin gave a bluish-green color with ferric chloride and ferric alum solutions. Madronin was precipitated by salts of the heavy metals of copper, mercury, silver, and lead. Madronin gave a positive Sanio-bichromate test. Madronin precipitated with the alkaloids or alkaloidal salts of atropine, berberine, brucine, caffeine, cinchonidine, cinchonine, hydrastine, and strychnine (3). It formed a precipitate with the organic bases pyridine and phenazone (6).

The following tests indicated the presence of a gallotannin and a catechol tannin. The formaldehyde test (7) gave the characteristic pink fluffy precipitate of a catechol tannin and upon the addition of solutions of ferrous hydroxide and sodium acetate the filtrate became violet, which further indicated the presence of gallotannin. When madronin was heated above 210° for 30 minutes, a gas evolved and a sublimate formed on the sides of the vessel. The gas gave a white precipitate when passed through lime water and the sublimate turned red with ferric chloride solution and blue with ferrous sulfate solution. These tests on the sublimate as well as a paper chromatogram indicated the presence of pyrogallol, resulting from the gallic acid of gallotannin. A precipitate formed upon the addition of ammonium sulfide and ammonium molybdate, which are positive tests for gallotannin. Likewise, madronin precipitated with bromine water, which is positive for catechol tannin. The precipitate which formed upon the addition of lead acetate solution was soluble in acetic acid; this is characteristic of a catechol tannin. Madronin gave a positive phlobaphene test (8), indicating the presence of catechol tannin. The pine wood test (lignin reaction) gave the characteristic pink (7), indicating the presence of phloroglucinol from catechol tannin.

Ultraviolet Absorption.—Although madronin was thought to be a mixture of several substances instead of a pure substance, its absorption spectra were examined for characteristic features and were compared to those of tannic acid N.F. The ultraviolet absorption spectrum of an aqueous solution containing 16 mcg./ml. madronin or tannic acid N.F. was determined from 210 mμ to 350 mμ at various pH from 4–11 using a Cary recording spectrophotometer. Acetate, phosphate, and boric acid–sodium hydroxide–potassium chloride mixtures were used as the corresponding buffer solutions to maintain the desired pH. The same conditions were followed as previously employed by Hammarlund, Pennington, and Rising (1). The positions of the peaks in the spectra obtained for tannic acid N.F. (Fig. 1) were nearly identical at each pH with those previously reported for madronin; however, their magnitudes were greater, as reported by Sohn (9). The only essential difference was the presence of a more distinct absorption peak at 230 mμ for tannic acid N.F. at pH 10. For simplicity in Fig. 1 only the spectra for tannic acid at pH 6, 8, and 10 are given.

Infrared Absorption.—The infrared spectrum of 2 mg. of madronin and "purified" madronin in a 400-mg. KBr pellet was measured with a Beckman infrared recording spectrophotometer (model IR-5A) from 2 μ to 16 μ. The position of the absorption peaks was practically identical to that of an equal quantity of tannic acid N.F. with only insignificant shifts in the peaks as seen in Fig. 2. The infrared absorption spectra for the two madronin samples were identical.

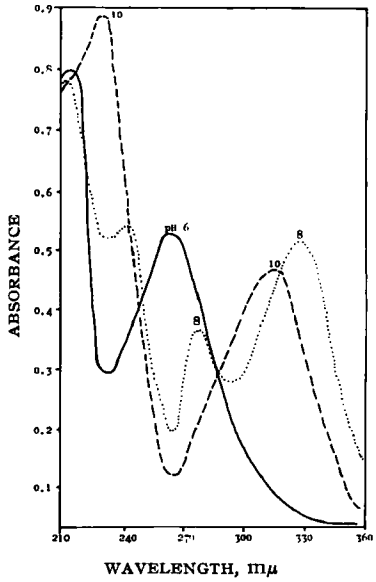

Fig. 1.—Ultraviolet absorption spectrum of tannic acid N.F. at various pH.

[2] Microbiology Department, University of Washington Medical School, Seattle.

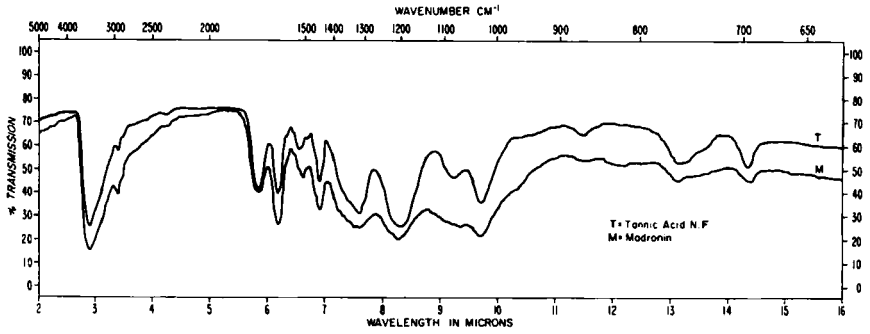

Fig. 2.—Infrared spectra of tannic acid N.F., madronin, and "purified" madronin. (The two madronin spectra are similar.)

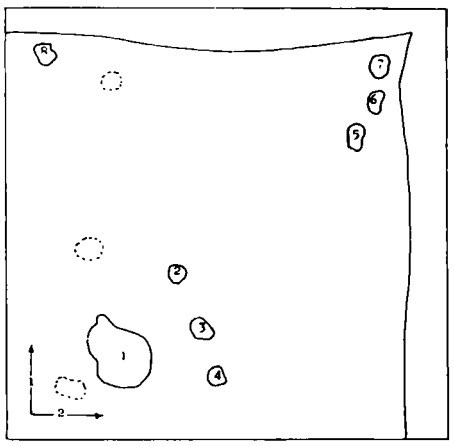

Fig. 3.—Two-dimensional chromatogram of madronin. Solvent system: first direction, 6% acetic acid; second direction, *n*-butanol:acetic acid:water (4:1:5). Reagent: 0.5% ammoniacal silver nitrate in methanol. Identified spots are (solid line): *1*, gallotannin; *2*, gallic acid; *3*, *m*-digallic acid; *4*, trigallic acid; *5*, catechin; *6*, phloroglucinol; *7*, catechol; *8*, glucose.

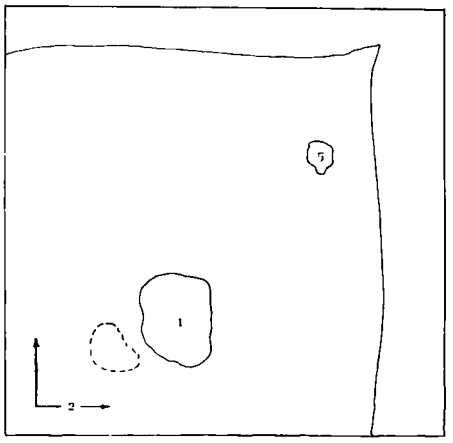

Fig. 4.—Two-dimensional chromatogram of "purified" madronin. Solvent system: first direction, 6% acetic acid; second direction, *n*-butanol:acetic acid:water (4:1:5). Reagent: 0.5% ammoniacal silver nitrate in methanol. Identified spots are: *1*, gallotannin; *5*, catechin.

Paper Chromatography.—The ascending filter paper partition chromatography of madronin using a water-saturated butanol solvent system on Whatman No. 1 paper previously reported by Hammarlund and associates (1) was repeated on tannic acid N.F. under identical conditions. Tannic acid also separated similarly into two antibacterially active spots.

The detailed chromatographic study of madronin and "purified" madronin was conducted on two-dimensional Whatman No. 1 chromatographic sheets, 14 in. square, by using the ascending method (4) and employing 6% acetic acid as the developing solvent in the first direction and *n*-butanol:acetic acid:water (4:1:5) in the second direction. The reagent employed for detecting the position of the spots was 0.5% ammoniacal silver nitrate in methanol (7, 10). The chromatogram of madronin is shown in Fig. 3, and the color of the spots of the various phenolic substances varied from brown to green-brown to blue-black. The following substances in madronin were identified by comparisons of the position and color of their spots with those given by known reagents used singularly or in combination: glucose, gallic acid, phloroglucinol, catechol, catechin, and tannic acid. In the case of gallotannin, *m*-digallic acid, and trigallic acid for which the pure reagents were not readily available, the relative position of the spots in each case were compared with similar chromatograms of tannins reported in the literature (4). This method likewise was used in determining the presence of protocatechuic acid and pyrogallol in the hydrolytic products. The spots encircled by dotted lines remain unidentified. Using the same method a chromatogram of "purified" madronin contained fewer spots (Fig. 4), showing only the presence of gallotannin and possibly catechin.

Hydrolysis of Madronin and Identification of the Hydrolytic Products.—One gram of madronin was acid-hydrolyzed by refluxing in 6% sulfuric acid for 20 hours. A two-dimensional paper chromatogram was made of the filtrate employing the same method described previously. The chromatogram is shown in Fig. 5 and the following spots were identified: gallic acid, catechin, phloroglucinol, catechol, glucose, and protocatechuic acid. A reasonably drastic acid treatment was made to obtain complete hydrolysis.

A 200-mg. sample of madronin was similarly alkali-hydrolyzed by dissolving it in an aqueous 5% sodium hydroxide solution which was shaken at room temperature for a few minutes. The resulting solution was chromatographed as previously described and the following spots were identified as shown in Fig. 6: gallotannin, gallic acid, *m*-digallic acid, trigallic acid, catechin, phloro-

glucinol, catechol, glucose, and protocatechuic acid. Since phloroglucinol and pyrogallol have the same R_f values for the two-dimensional solvents used, it was difficult to prove conclusively whether pyrogallol was there when phloroglucinol was present (10).

One-half gram of "purified" madronin was dissolved in 2% hydrochloric acid and kept at 95° for 1 hour. The resulting solution was then spotted on paper and two-dimensional chromatography was carried out (Fig. 7). By comparison with the chromatogram of "purified" madronin before hydrolysis (Fig. 4) which contained essentially

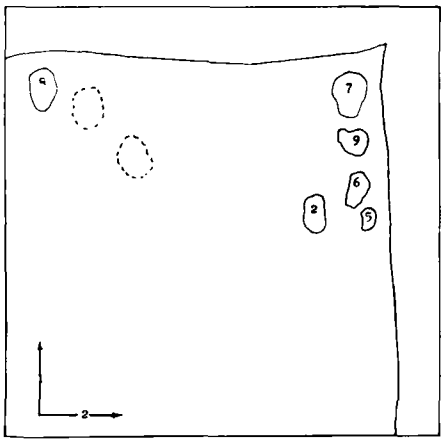

Fig. 5.—Two-dimensional chromatogram of acid-hydrolyzed madronin. Solvent system: first direction, 6% acetic acid; second direction, *n*-butanol:acetic acid:water (4:1:5). Reagent 0.5% ammoniacal silver nitrate in methanol. Identified spots are (solid line): *2*, gallic acid; *5*, catechin; *6*, phloroglucinol; *7*, catechol; *8*, glucose; *9*, protocatechuic acid. Length of hydrolysis: 20 hours with 6% sulfuric acid.

gallotannin and possibly catechin, the following additional hydrolytic products were obtained: gallic acid, *m*-digallic acid, trigallic acid, catechol, protocatechuic acid, and glucose. The hydrolysis was still incomplete because some gallotannin remained. Only a relatively mild acid treatment was required to show that "purified" madronin was hydrolyzable.

DISCUSSION

In the original study by Hammarlund, Pennington, and Rising (1) madronin was reported as being a mixture of unknown antibacterially active compounds. The current study revealed that the active principles were essentially gallotannin and catechol tannin in addition to some of the decomposition products of the tannins and that all of the results and observations made by the previous workers could be interpreted now on the basis of the newly determined composition of madronin.

The antibacterial activity of tannins has been extensively studied (11–16). In this present study madronin possessed slightly less antibacterial activity than tannic acid N.F. This might be due to the presence in madronin of the decomposition products of tannic acid, such as catechol, phloroglucinol, catechin, gallic acid, and glucose in addition to nonhydrolyzed gallotannin and catechol tannin. "Purified" madronin which did not contain the decomposition products showed greater antibacterial activity than did the original madronin but still not quite as much as did tannic acid N.F.

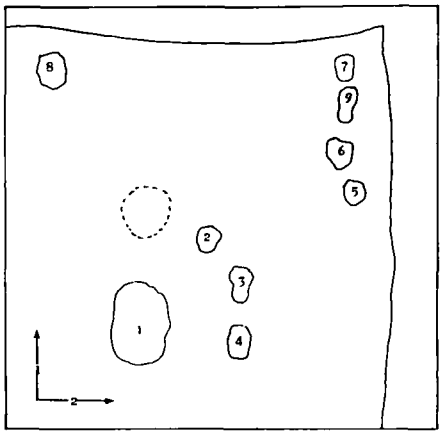

Fig. 6.—Two-dimensional chromatogram of alkali-hydrolyzed madronin. Solvent system: first direction, 6% acetic acid; second direction, *n*-butanol:acetic acid:water (4:1:5). Reagent: 0.5% ammoniacal silver nitrate in methanol. Identified spots are (solid line): *1*, gallotannin; *2*, gallic acid; *3*, *m*-digallic acid; *4*, trigallic acid; *5*, catechin; *6*, phloroglucinol; *7*, catechol; *8*, glucose; *9*, protocatechuic acid. Length of hydrolysis: 2 minutes with 5% sodium hydroxide solution.

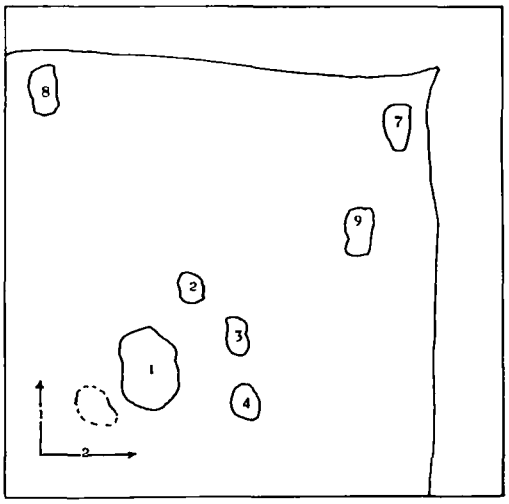

Fig. 7.—Two-dimensional chromatogram of acid-hydrolyzed "purified" madronin. Solvent system: first direction, 6% acetic acid, second direction, *n*-butanol:acetic acid:water (4:1:5). Reagent: 0.5% ammoniacal silver nitrate in methanol. Identified spots are (solid line): *1*, gallotanin; *2*, gallic acid; *3*, *m*-digallic acid; *4*, trigallic acid; *7*, catechol; *8*, glucose; *9*, protocatechuic acid. Length of hydrolysis: 1 hour with 2% hydrochloric acid.

The physical properties of madronin (yellow color, amorphous structure, and decomposition at 200°) are closely parallel to those of tannins. The levorotation of madronin is possibly due to the presence of catechol tannin which masked the usual dextrorotation of gallotannin. The loss of anti-

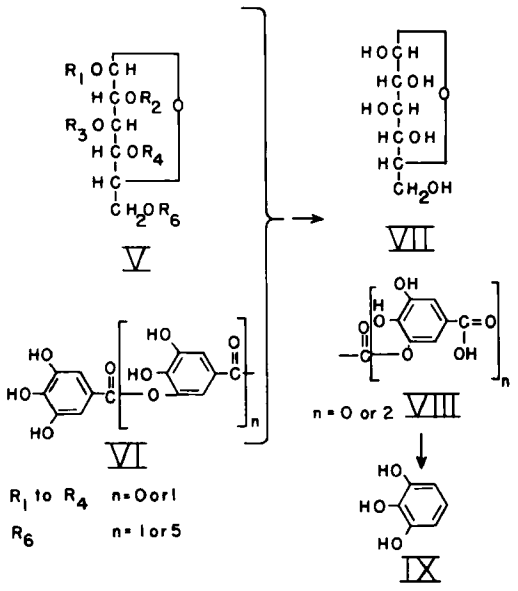

Fig 8—Degradation of gallotannin.

bacterial activity of madronin above pH 7 is due to the fact that tannins are less stable in alkaline solutions (3).

It was largely the fact that the qualitative chemical tests of madronin were similar to the reactions given by the various polyhydroxy phenolic constituents of tannic acid which led to the recognition that madronin was probably a tannin, particularly since no other antibacterial component was found present. Although various polyhydroxy compounds may give some of the individual chemical reactions, all the tests are given only by tannins (5).

The evidence that one of the tannins was gallotannin (V–VI, Fig. 8) was substantiated by the presence of gallic acid (VIII, n = 0) and glucose (VII). Tests for the presence of glucose in madronin, such as Molisch or yeast fermentation, were negative; however, glucose was identified from the hydrolytic products following purification (3) by both chemical and biological tests (positive osazones, Fehling's, Tollen's, yeast fermentation) and by paper chromatographic methods (Figs. 5, 6, and 7). Gallic acid and its decomposition product, pyrogallol (IX), were identified by their characteristic chemical tests and by paper chromatography.

The evidence that a catechol tannin (I, Fig. 9) was also present in madronin was obtained through the following series of reactions—the formation of a bluish-green color with iron compounds, the precipitation with bromine solution and formaldehyde solution, and formation of the red phlobaphene and a positive pine wood test which indicated the presence of phloroglucinol (II, Fig. 9). Finally, the identification by paper chromatography of protocatechuic acid (IV, Fig. 9) among the hydrolytic products of madronin is further evidence for the presence of catechol tannin.

Additional decomposition products of tannins such as catechol (III, Fig. 9), m-digallic acid (VIII, Fig. 8, n = 1), and trigallic acid possibly (VIII, Fig. 8, n = 2) were also identified by paper chromatography (Figs. 5, 6, and 7).

Because all evidence showed madronin to be a mixture of gallotannin and catechol tannin which hydrolyzed readily, the proportion of the various phenolic constituents and other hydrolytic products may vary greatly during extraction and purification. In addition, the normal seasonal variation could influence the concentration of the active tannin principle. Therefore, it is reasonable to assume that the yield of madronin and the antibacterial assays will show large variations depending upon the history of the sample tested.

One of the general observations which has resulted from this study is the reiteration of the importance of tannins as antibacterial substances in higher plants. Because of their natural wide occurrence in plants it is highly probable that in some cases the activity found in the higher plants during the course of the many surveys for antibacterial agents as reviewed recently by Nickell (17) might be due partly or entirely to the presence of tannins. Therefore, if antibacterial activity is found in extracts of higher plants, the extracts should be carefully tested for the presence of tannins.

SUMMARY AND CONCLUSIONS

A simple modified laboratory extraction and purification procedure was developed for the extraction of more antibacterially active madronin than was obtained previously.

Experimental evidence involving studies of light absorption, paper chromatography, qualitative chemical tests, and microbiological fermentation experiments shows that madronin is a mixture of gallotannin and catechol tannin and their various decomposition products of gallic acid, m-digallic

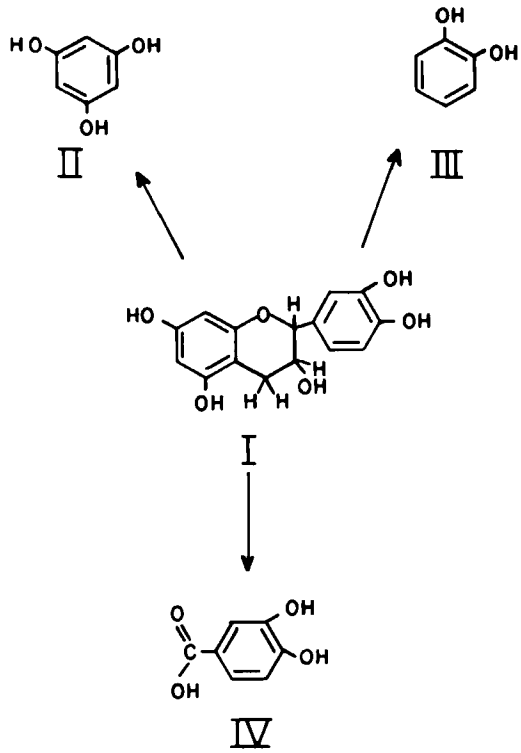

Fig. 9.—Degradation of catechol tannin (catechin).

acid, trigallic acid, catechol, phloroglucinol, and glucose.

Tannin has been shown to be a rather potent antibacterial substance; its possible influence upon the various surveys for the presence of antibacterial agents in higher plants should be investigated during the surveys.

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Colorimetric Method for Determination of Uracil Mustard and Related Alkylating Agents

By H. G. PETERING and G. J. VAN GIESSEN

A colorimetric method for uracil mustard has been developed which is equally useful for other aromatic and aliphatic nitrogen mustard antitumor drugs. This method has been used to determine these drugs in plasma pools and for the study of their kinetics of hydrolysis and reactivity with nucleophilic reagents.

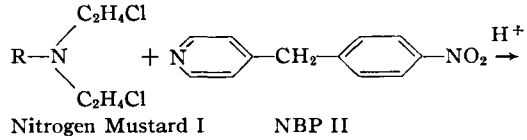
SEVERAL COLORIMETRIC methods for the determination of specific nitrogen mustards have been described by Klatt, Griffin, and Stahlin (1), Friedman and Boger (2) and Ausman, Crevar, Hagedorn, Bardos, and Ambrus (3). These methods all are based on the method for alkylating agents first reported by Epstein, Rosenthal, and Ess (4) which involves reaction of these agents with 4-(*p*-nitrobenzyl) pyridine. A photofluorometric method for ethylenimino and chloroethylamino groups has also been devised by Mellett and Woods (6). This latter method has the advantage of extreme sensitivity and thus may be useful for blood-level studies; it has the disadvantage of possible large losses because of involved and laborious manipulations.

The colorimetric methods are simpler than the fluorometric method and are useful in kinetic studies and in following the levels of these drugs when they are used in surgical perfusion studies. However, when we tried them with uracil mustard, 5-bis(2-chloroethyl) aminouracil, a number of difficulties were encountered. Since the method of Mellett and Woods was not found suitable for our purposes, we decided to attempt a modification of the colorimetric method of Klatt, Griffin, and Stahlin (1) so that a variety of nitrogen mustards could be determined.

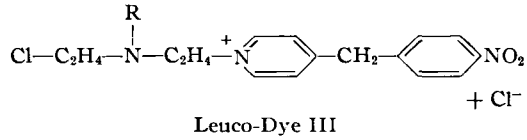
This method which is equally useful with aliphatic, aromatic, and heterocyclic nitrogen mustards and which can be conveniently used to determine these antitumor agents in perfusion blood pools, and on tissue extracts, is presented here.

EXPERIMENTAL

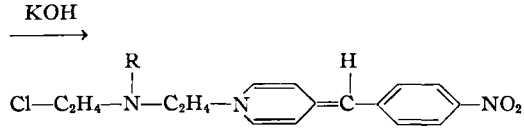
The reaction of alkylating agents with 4-(*p*-nitrobenzyl) pyridine (NBP) (II), which produces the chromophore, is carried out in our method under conditions similar to those originally described by Epstein, *et al.* (4). In the reaction formulated in Eq. 1, the solubility of the reaction product III is dependent on whether R is alkyl, aryl, or heterocyclic. Similarly the color formed from the chromophore IV when alkali is added will depend on the nature of R. In addition, Epstein, *et al.* (4), showed that the type of alkali used is of importance in the intensity of color formation. All of these matters were carefully considered in formulating the method outlined below.



Nitrogen Mustard I NBP II



Leuco-Dye III



Chromophore IV

The method worked out for uracil mustard (*Ia*) was found to give good results for dopan (*Ib*), chlorambucil (*Ic*), tris-chloroethylamine hydrochloride (*Id*), and HN-2 (Merck Mustargen) (*Ie*). The structures of these compounds, all of which are used as antitumor agents, are given in Table I.

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Colorimetric Method for Determination of Uracil Mustard and Related Alkylating Agents

By H. G. PETERING and G. J. VAN GIESSEN

A colorimetric method for uracil mustard has been developed which is equally useful for other aromatic and aliphatic nitrogen mustard antitumor drugs. This method has been used to determine these drugs in plasma pools and for the study of their kinetics of hydrolysis and reactivity with nucleophilic reagents.

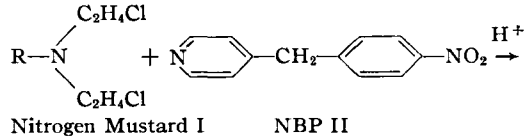
SEVERAL COLORIMETRIC methods for the determination of specific nitrogen mustards have been described by Klatt, Griffin, and Stahlin (1), Friedman and Boger (2) and Ausman, Crevar, Hagedorn, Bardos, and Ambrus (3). These methods all are based on the method for alkylating agents first reported by Epstein, Rosenthal, and Ess (4) which involves reaction of these agents with 4-(*p*-nitrobenzyl) pyridine. A photofluorometric method for ethylenimino and chloroethylamino groups has also been devised by Mellett and Woods (6). This latter method has the advantage of extreme sensitivity and thus may be useful for blood-level studies; it has the disadvantage of possible large losses because of involved and laborious manipulations.

The colorimetric methods are simpler than the fluorometric method and are useful in kinetic studies and in following the levels of these drugs when they are used in surgical perfusion studies. However, when we tried them with uracil mustard, 5-bis(2-chloroethyl) aminouracil, a number of difficulties were encountered. Since the method of Mellett and Woods was not found suitable for our purposes, we decided to attempt a modification of the colorimetric method of Klatt, Griffin, and Stahlin (1) so that a variety of nitrogen mustards could be determined.

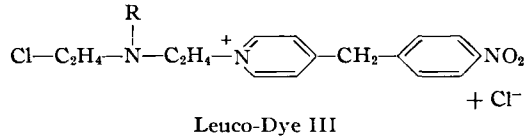
This method which is equally useful with aliphatic, aromatic, and heterocyclic nitrogen mustards and which can be conveniently used to determine these antitumor agents in perfusion blood pools, and on tissue extracts, is presented here.

EXPERIMENTAL

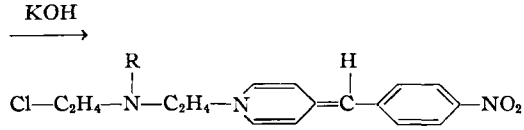
The reaction of alkylating agents with 4-(*p*-nitrobenzyl) pyridine (NBP) (II), which produces the chromophore, is carried out in our method under conditions similar to those originally described by Epstein, *et al.* (4). In the reaction formulated in Eq. 1, the solubility of the reaction product III is dependent on whether R is alkyl, aryl, or heterocyclic. Similarly the color formed from the chromophore IV when alkali is added will depend on the nature of R. In addition, Epstein, *et al.* (4), showed that the type of alkali used is of importance in the intensity of color formation. All of these matters were carefully considered in formulating the method outlined below.



Nitrogen Mustard I NBP II



Leuco-Dye III



Chromophore IV

The method worked out for uracil mustard (*Ia*) was found to give good results for dopan (*Ib*), chlorambucil (*Ic*), tris-chloroethylamine hydrochloride (*Id*), and HN-2 (Merck Mustargen) (*Ie*). The structures of these compounds, all of which are used as antitumor agents, are given in Table I.

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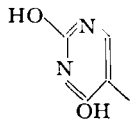
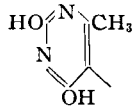
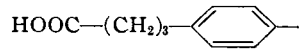
TABLE I.—STRUCTURE OF NITROGEN MUSTARDS INVESTIGATED WITH GENERAL FORMULA R—N $(CH_2—CH_2Cl)_2$

	Trade Name	Chemical Name	R Group
I*a*	Uracil mustard	5-Bis-(2-chloroethyl)-aminouracil	
I*b*	Dopan	5-Bis-(2-chloroethyl)-amino-6-methyluracil	
I*c*	Chlorambucil	4′-Bis-(2-chloroethyl)-amino-3-phenylbutyric acid	
I*d*	HN-3	Tris-(2-chloroethyl)-amine - 1·HCl	$Cl—CH_2—CH_2—$
I*e*	HN-2	Bis-(2 chloroethyl)-methylamine · HCl	$CH_3—$

Apparatus.—A Beckman B spectrophotometer was used throughout this work, but any other suitable instrument can be used. The color developed in this method was most accurately determined at 600 mμ.

Reagents.—Five per cent of 4-(*p*-nitrobenzyl) pyridine (NBP) obtained from Aromil Chemical Co., Baltimore, Md., was dissolved in acetone and used as the chromophore-producing reagent. Potassium hydroxide (1*N*) in 90% ethanol was used as the base for color development.

Nitrogen Mustard Solutions.—Uracil mustard, dopan, chlorambucil, and HN-3 were synthesized in the Upjohn Laboratories; HN-2 was obtained as Mustargen from Merck and Co. Stock solutions of the first four compounds were prepared by dissolving 10 mg. of each drug in 5 ml. of dimethylacetamide (DMA) and diluting these solutions to 100 ml. with absolute ethanol or saline. (The drugs are more stable if the dilution is made in absolute ethanol.) A stock solution of Mustargen, which is a mixture of 10 mg. of HN-2 and 90 mg. of sodium chloride, was prepared by dissolving 100 mg. in 10 ml. of water and then diluting 1 ml. of this solution with 0.5 ml. of DMA and enough absolute ethanol or saline to bring the volume to 10 ml. Thus each stock solution contained the same weight concentration of drug and the same dilution of DMA. Aliquots of these stock solutions were used to obtain the standard curves and to explore the recovery of uracil mustard from plasma.

Buffer Solutions.—Phthalate buffer of pH 4.0 was prepared according to Clark (5), and the acetate buffer of pH 4.6 according to the directions of Friedman and Boger (2).

PROCEDURES

Standardization Curves.—An aliquot of stock solution of I*a*, I*b*, I*c*, I*d*, or I*e* in the range of 10–70 mcg. was placed in a 12-ml. centrifuge tube. To this was added 1.0 ml. of pH 4.0 phthalate buffer, 1.0 ml. of NBP reagent, 1.0 ml. of 0.9% saline, and enough absolute ethanol to bring the volume to 4.0 ml. This mixture with a boiling chip was then heated for 20 minutes in a water bath at 80°. A blank without the mustard but with 1.0 ml. of absolute ethanol was treated in the same way.

After the heating period, during which the condensation was completed, the tubes were plunged into an ice bath and after being cooled were individually treated for maximum color formation and subsequent spectrophotometric analysis at 600 mμ. The contents of each tube were carefully transferred to a 5-ml. volumetric flask, 0.1 ml. of 1.0 *N*

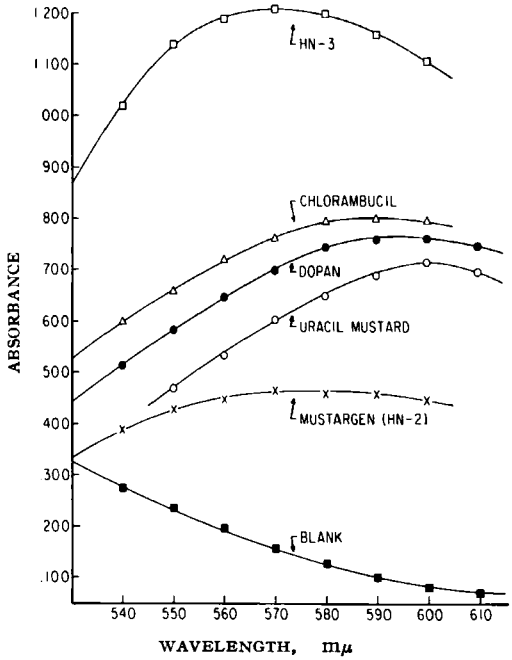


Fig. 1.—Ultraviolet absorption curves of chromophores produced by reaction of NBP reagent with several nitrogen mustards.

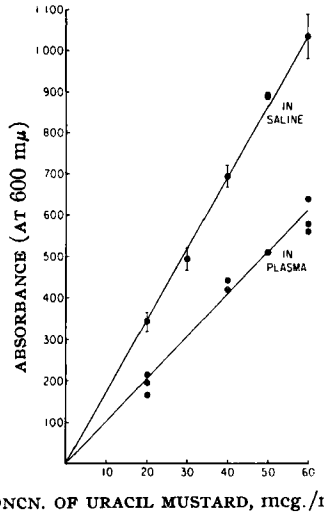


Fig. 2.—Beer's law plot of uracil mustard in saline compared with similar amounts extracted from plasma. These data indicate the extent of decomposition or binding of uracil mustard which can be expected in determining the concentration of this drug in blood.

KOH was added, and the volume brought to 5.0 ml. with ethanol. Each flask was thoroughly mixed, and placed in a cell for spectral analysis. The absorbance was observed to decrease at the rate of 1% per minute after the addition of the alkali, making it important to read the color at a designated time interval after mixing the reagents. All of our data for standard curves were obtained by making the readings within 2–3 minutes after the addition of alkali. Under these conditions a plot of absorbance *versus* concentration of drug gave a linear relationship (Beer's law) up to 70 mcg. in all instances.

Recovery of Uracil Mustard from Blood Plasma.—The following procedure was used to determine the recovery of uracil mustard (I*a*) from blood plasma. To heparinized rat plasma, uracil mustard was added from a DMA-saline stock solution in amounts so that 1.0 ml. of plasma contained 20–60 mcg. of I*a*. One milliliter of such plasma was immediately extracted with 2.0 ml. of acetone, making certain that thorough mixing and extraction occurred. The precipitated proteins were removed by centrifugation and the extract saved. The precipitate was extracted twice more with 1.0 ml. of acetone. The combined extract was then cooled in an ice bath and again centrifuged to remove residual protein. To this extract, in a 40-ml. centrifuge tube, was added 1.0 ml. of NBP reagent, 1.0 ml. of pH 4.0 phthalate buffer, and several milliliters of ethanol to control foaming.

This mixture was then heated at 60° for 5 minutes and at 80° for 20 minutes, during which the desired condensation occurred and acetone, which can react in the final color development, is removed. The resultant reaction product was then treated for color formation as described above. The chromophore produced from the drug extracted from plasma fades more rapidly than it did in the case of the standard curve investigation, probably because of the presence of unidentified soluble reactants. The rate of decrease of absorbance is about 5% per minute which makes it imperative that the readings be made quickly after the addition of alkali.

RESULTS

One of the important problems which must be resolved in the colorimetric determination of nitrogen mustards by the use of NBP reagent is the selection of the wavelength (or band) for maximum sensitivity of color determination. This varies enough for different nitrogen mustards that it accounts for some of the differences in the methods now in use. Our data in Fig. 1 indicate that the selection of 600 mμ, the maximum for the uracil mustard color, is satisfactory for the other compounds tested, although each has a somewhat different maximum. However, if 562 mμ had been selected as indicated in the work of Klatt, *et al.* (1), and 540 as indicated by Friedman and Boger (2), the results with uracil mustard (I*a*), dopan (I*b*), and chlorambucil (I*c*) would have been lower and thus decreased the sensitivity of the test.

Since excellent linear plots of optical density *versus* concentration (Fig. 2) were obtained in every case, the optical density constants for 10 mcg. per ml. are compared in Table II. These data indicate the applicability of the method to a wide variety of nitrogen mustards with comparable sensitivity in all cases except that of HN-2, which was obtained as Mustargen (Merck). Since Mustargen is a mixture of HN-2 and sodium chloride, no real basis for purity or lack of decomposition was available and it is likely that the lower results reflect less HN-2 available in the sample. Nevertheless, the method gave excellent agreement with Beer's law; thus the color with HN-2 by this method is consistent and determinable.

In all reports on the reaction of NBP with nitrogen mustards, the pH of the reaction mixture has been cited as critical; indeed it is important to have an acidic medium present as the reaction kinetics have shown. Epstein, *et al.* (4), indicated a phthalate buffer at pH 4.0 was the best for the original study. Klatt, *et al.* (1), stated that the pH should be between 4.5 and 5.0 and attempted to achieve this by adding acetic acid to the mixture before heating. Friedman and Boger (2) found acetate buffer at pH 4.6 to be better than phthalate buffer at the same

TABLE II.—ABSORBANCE OF CHROMOPHORES DEVELOPED WITH DIFFERENT NITROGEN MUSTARDS

	Mol. Wt.	Eq. Wt.	Absorbance at 600 mμ: Per 10 mcg. of Drug ± S.E.	Absorbance at 600 mμ: Per 0.1 μm. of Drug
Uracil mustard	252	126	0.173 ± 0.007	0.437
Dopan	266	133	0.185 ± 0.007	0.492
Chlorambucil	304	152	0.199 ± 0.006	0.605
HN-3 HCl	241	80	0.282 ± 0.014	0.679
HN-2 HCl (Mustargen)	192.5	96	0.117 ± 0.003	0.223

pH. We have compared the color developed with Ia and Id when acetate buffer of pH 4.6 and phthalate buffer of pH 4.0 are used. Our results show that the original work of Epstein, *et al.* (4), is valid, and that a phthalate buffer of pH 4.0 gives considerably higher values. We therefore held to the original buffer selection.

Potassium hydroxide (1.0 *N*) in 90% ethanol was used for final color development instead of either K_2CO_3 solutions or triethylamine as suggested by Epstein, *et al.* (4), and Klatt, *et al.* (1). Thereby we have avoided the problems of solubility of K_2CO_3 when acetone or ethanol are present and the critical nature of the water concentration as well as the difficulties which are inherent in safely handling triethylamine and in keeping it stabilized. This modification contributed greatly to the suitability of the method for both aqueous-soluble and aqueous-insoluble nitrogen mustard derivatives.

In other studies we found that DMA is not only a useful solvent for making up stock solutions of uracil mustard and other alkylating agents, but also that its presence at 2–5% in a saline solution of these drugs increase their stability. From our data DMA at levels up to 10% in the solutions of any of the mustards investigated does not affect the chromophore development or the subsequent color formation. Therefore, nitrogen mustard solutions containing DMA may be prepared for kinetic or biological studies without fear of altering the analytical data when this method is used.

The addition of uracil mustard in DMA-saline or ethanol-saline solutions to plasma and the immediate analysis of this mixture according to the method described above gave consistent values of about 60% recovery of the added drug. These data which are shown in Fig. 2 compared with the standard curve in the absence of plasma indicate the rapid inactivation of the drug or its complexing with plasma proteins. When trichloracetic acid precipitation of plasma protein and extraction of uracil mustard were attempted, no color development at all was obtained. Similarly when proteins were precipitated by boiling for 3 minutes in the presence of hydrochloric acid according to the method of Friedman and Boger (2) no evidence of uracil mustard in the extract was obtained. When, however, the method of Klatt, *et al.* (1), was used and the plasma was added directly to the reaction mixture, color development of intensity similar to that found by our method was obtained, although the consistency of the results was not satisfactory. Thus it is evident that uracil mustard is affected by the methods for removal of plasma protein.

DISCUSSION

Of the colorimetric methods mentioned earlier for the determination of nitrogen mustard drugs useful in cancer chemotherapy, only that of Klatt, Griffin, and Stahlin (1) has been shown to be suitable for both aromatic and aliphatic compounds. This method, however, has several shortcomings which the present method avoids. Since the pH of the reaction medium for the production of the chromophore is critical, we have chosen to use a definite buffer—phthalate buffer at 4.0—rather than adjusting the acidity with glacial acetic acid as Klatt, *et al.*, did. Furthermore, we have eliminated the use of triethylamine as the alkali for color development to avoid handling a highly toxic reagent which is unstable and requires frequent purification and have substituted instead ethanolic potassium hydroxide which is more readily available and easier to handle.

We have avoided the use of methods for extracting the drugs from plasma which will destroy the drug as Klatt, *et al.*, also attempted to do. We, however, prefer the extraction of the drug from plasma with acetone in which it is stable, although we have used the method of Klatt, *et al.*, with some success. We have found that uracil mustard is unstable when trichloracetic acid is used for extraction purposes or when the plasma is heated to boiling in the presence of hydrochloric acid as Friedman and Boger (2) recommended.

Only the method of Mellett and Woods (6) offers advantages over that described here in that it is more sensitive because of the use of a fluorometric method. Our experience with this method indicated to us that in its present form it was not suitable for routine use by laboratory technicians. In addition, we feel that the results with uracil mustard by this method as reported recently by Mellett and Woods (6) indicate very great losses during the laborious manipulations which involve the use of strong hydrochloric acid, known to cause destruction of uracil mustard. Nevertheless, if a more sensitive method is required than that described here, fluorometric methods of analysis should be investigated and developed.

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Emulsifying Effects of Several Ionic Surfactants on a Nonaqueous Immiscible System

By J. D. McMAHON, R. D. HAMILL, and R. V. PETERSEN

The emulsifying effects of twelve anionic and five cationic surfactants on the nonaqueous immiscible system, glycerin and olive oil, were studied. Three anionic agents, dioctyl sodium sulfosuccinate, diamyl sodium sulfosuccinate, and calcium stearate, and two cationic agents, benzalkonium chloride and lauryltrimethylammonium bromide, failed to stabilize the system. On the other hand, four anionic agents, tetrasodium-N-(1,2-dicarboxyethyl)-N-octadecyl-sulfosuccinamate, sodium lauryl ether sulfate, sodium lauryl sulfate, and sodium stearate, and three cationic agents, stearyltrimethylammonium chloride, cetylpyridinium chloride, and stearyldimethylbenzylammonium chloride, produced stable emulsions. The anionic agents formed by the saponification of the fatty acids present in the olive oil with 2-amino-2-methyl-1,3-propanediol, tris (hydroxymethyl) aminomethane, triethanolamine, ethanolamine, or ammonia, yielded clear, stable emulsions. Emulsification occurred more readily, or preferentially, when the surfactant was first added to the glycerin. All of the emulsions formed were of the oil-in-glycerin type, except those containing sodium stearate or stearyltrimethylammonium chloride.

DURING RECENT YEARS rapid advances have been made in the technology of surface-active agents (surfactants). A multitude of new agents have been developed and their properties and actions studied. These studies have resulted not only in a vast expansion of the knowledge of the better known actions, but also in the discovery of new actions and new applications for these agents.

Surfactants exhibit many properties, among which are included emulsifying, demulsifying, wetting, foaming, antifoaming, dispersing, solubilizing, detergent, and spreading actions. Many of these actions are interrelated and all are manifestations of the thermodynamic function of surfactants in effecting a reduction in the surface or interfacial tension between a liquid and an adjoining phase. The amphiphilic nature of surfactant molecules results in a simultaneous attraction for both polar and nonpolar substances which accounts for some of their actions.

The actions enumerated above find applications in a large number of industries, including the pharmaceutical and cosmetic industries. Many pharmaceutical applications have been extensively investigated. For example, the use of surfactants as emulsifying agents for oil-water systems, as detergents, dispersing agents, suppository bases, disinfectants, preservatives, and fecal softeners, is well established. There are also reports in the literature regarding the use of surfactants as solubilizing agents for volatile oils (1), phenobarbital in a nonalcoholic vehicle (2), reserpine (3), benzoic acid derivatives (4), hexachlorophene (5, 6), and many other substances for internal and external application. Many studies also have been performed and reviewed on the effects of surfactants on gastrointestinal absorption of drugs (7–9), absorption of fats (7), and percutaneous absorption (10). Despite the abundant literature regarding the effects of surfactants on aqueous and aqueous-oil systems, relatively little is mentioned on nonaqueous, binary, immiscible systems, such as glycerin and olive oil. For this reason, and because these solvents are pharmaceutically useful, these agents were selected as a model for the study of the effects of surfactants on such a system. The results obtained in the descriptive phase of this investigation provide the basis for this report.

METHODS

The system employed in each experiment consisted of equal weights of glycerin and olive oil with a variable concentration of surfactant. The glycerin and olive oil used were U.S.P. grade. Every reasonable precaution was taken to avoid undue exposure of these reagents to the atmosphere, except during actual manipulation; although, due to the hygroscopicity of glycerin, anhydrous conditions would have been almost impossible to achieve and maintain under the experimental conditions employed.

Two methods of mixing were used. In Method I the surfactant was weighed and added directly to a weighed quantity of glycerin. This mixture was then transferred to a glass mortar. The oil was added slowly in small portions, with rapid trituration after each addition. In Method II the requisite quantity of surfactant was added to the oil. The oil and surfactant were then transferred to a glass mortar and the glycerin was added slowly, in small portions, with vigorous trituration after each addition.

Liquid surfactants were weighed directly into a tared beaker which contained the requisite weight

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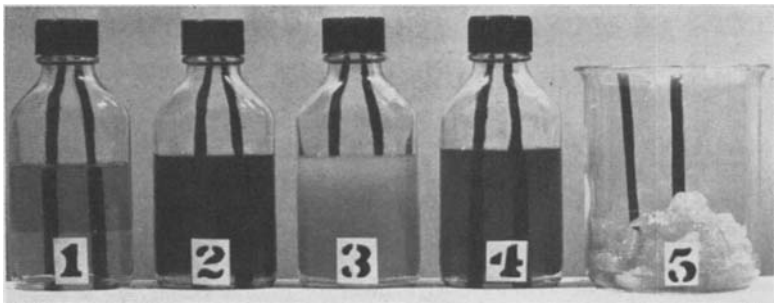

Fig. 1.—*1*, A typical separation; *2*, a partial separation; *3*, a somewhat opaque emulsion containing 3% Aerosol 22; *4*, a clear emulsion containing 1% triethanolamine; *5*, a semisolid emulsion containing 10% sodium lauryl sulfate.

of glycerin (Method I) or olive oil (Method II). Insoluble surfactants were incorporated into the glycerin or oil by warming the mixture on a water bath until solution or intimate mixing was effected. The warm mixtures were allowed to cool to room temperature before being used.

In some cases it was advantageous to make stock solutions of surfactants in glycerin or oil. For

TABLE I.—EMULSIFYING EFFECT OF ANIONIC SURFACTANTS ON EQUAL WEIGHTS OF GLYCERIN AND OLIVE OIL

Agent	%	Method I[a]	Method II[a]	Emulsion, Appearance
Tetrasodium-N-(1,2-dicarboxyethyl)-N-octadecyl-sulfosuccinamate[b]	1.0	Emulsification	...	Opaque
	3.0	Emulsification	Separation	Opaque
	5.0	Emulsification	...	Opaque
Dioctyl sodium sulfosuccinate[c]	1.0	Separation[d]	Separation[d]	...
	3.0	Separation[d]	Separation[d]	...
Diamyl sodium sulfosuccinate[e]	1.0	Separation[d]	Separation[d]	...
	3.0	Separation[d]	Separation[d]	...
Sodium lauryl ether sulfate[f]	2.0	Emulsification	...	Opaque
	5.0	Emulsification	Separation	Opaque
Sodium lauryl sulfate	2.0	Partial sep.[d]	Separation	...
	5.0	Partial sep.[d]	Separation	...
	10.0	Emulsification	Separation	Opaque, semisolid
Sodium stearate	2.0	Emulsification[g]	Separation	Opaque, semisolid
Calcium stearate	2.0	Separation[g]	Separation[g]	...
	5.0	Separation[g]	Separation[g]	...
2-Amino-2-methyl-1,3-propanediol[h]	0.05	Separation[d]	...	...
	0.1	Separation[d]	...	...
	0.15	Emulsification	...	Clear
	0.25	Emulsification	...	Clear
	0.5	Emulsification	Separation	Clear
	1.0	Emulsification	Separation	Clear
	2.0	Emulsification	Separation	Clear
Ammonia (gas)	0.01	Emulsification	...	Clear
	0.025	Emulsification	...	Clear
	0.05	Emulsification	...	Clear
	0.1	Emulsification	Separation	Clear
	0.15	...	Separation	...
	0.2	...	Emulsification	Clear
	0.25	Emulsification	Emulsification	Clear
	0.3	...	Emulsification	Clear
	0.5	Emulsification	...	Clear
	1.0	Emulsification	...	Clear
Ethanolamine	2.0	Partial sep.[d]	Separation	...
	5.0	Partial sep.[d]	Separation	...
	10.0	Emulsification	Separation	Opaque, semisolid
Triethanolamine	0.063	Separation[d]	Separation	...
	0.125	Separation[d]	Separation	...
	0.25	Separation[d]	Separation	...
	0.5	Emulsification	Separation	Clear
	1.0	Emulsification	Separation	Clear
	2.0	Emulsification	Separation	Clear
	3.0	Emulsification	Separation	Clear
	4.0	Emulsification	Separation	Clear
	5.0	Emulsification	Separation	Clear
Tris (hydroxymethyl) aminomethane[h]	0.1	Emulsification	...	Clear
	0.25	Emulsification	...	Clear
	0.5	Emulsification	...	Clear
	1.0	Emulsification	Separation	Clear

[a] See text. Marketed as Aerosol 22 by American Cyanamid Co., Pearl River, N. Y. [c] Marketed as Aerosol OT by American Cyanamid Co., Pearl River, N. Y. [d] Emulsification was apparent by macroscopic and microscopic examination, but separation was detectable within 7 days. [e] Marketed as Aerosol AY by American Cyanamid Co., Pearl River, N. Y. [f] Marketed as Sipon ES by American Alcolac Corp. [g] Surfactant not soluble; dispersion formed on heating. [h] Commercial Solvents Corp.

example, to expedite the use of ammonia as a saponifying agent, stock solutions were prepared by bubbling the anhydrous gas into weighed quantities of glycerin and of olive oil. Requisite quantities of these stock solutions were used for the preparation of solutions of various concentrations.

Hand methods of trituration were employed throughout the experiment. A colloid mill and a hand homogenizer were used to compare the results obtained by hand methods, but these devices did not improve significantly the products obtained by hand trituration.

Three criteria were used to evaluate the results of manipulations by Methods I and II. The product was considered to be *stable* if emulsification occurred and there was no separation into layers for at least 7 days. The term *emulsions* is used to designate those products which did not separate into distinct layers, although solubilization may have contributed to the overall phenomenon. Figure 1 depicts the appearance of a completely separated product, a partially separated product, a clear emulsion, an opaque emulsion and a semisolid emulsion, all of which were aged for at least 1 week. Only the latter three examples were considered as emulsions. Another criterion was based on the *miscibility* of the emulsion with added olive oil or glycerin upon gentle stirring. This procedure was employed to determine emulsion type, based on the assumption that additional liquid of the external phase, but not of the internal phase, could easily be mixed with the emulsion. Finally, *microscopic* determination of emulsion type was facilitated by the preparation of a 1.0% solution of D & C red No. 18 dye in olive oil. Heat was applied to effect solution. The cooled oil, containing the dye, was subsequently mixed with glycerin and surfactants in the usual manner and the resulting emulsions examined microscopically.

To determine the influence of temperature on the miscibility of olive oil and glycerin, mixtures of these agents were prepared in increments of 5.0% ranging from 5.0% olive oil and 95.0% glycerin to 95.0% olive oil and 5.0% glycerin. Miscibility was not effected in any of these mixtures at temperatures up to 180°, at which point there was evidence of decomposition.

RESULTS

Anionic Agents.—Table I summarizes the results obtained using the anionic surfactants and the saponifying amines.

Cationic Agents.—Table II summarizes the results obtained using the cationic agents.

An examination of Table I reveals that stable emulsions were obtained from the use of tetrasodium - N - (1,2 - dicarboxyethyl) - N - octadecylsulfosuccinamate, sodium lauryl ether sulfate, sodium lauryl sulfate, sodium stearate, and from the saponifying amines, 2-amino-2-methyl-1,3-propanediol, tris(hydroxymethyl) aminomethane, triethanolamine, ethanolamine, and ammonia. Other agents produced emulsions which remained stable for several hours or days, but separated partially or completely on standing. For example, emulsification was observed immediately after manipulation of products containing dioctyl sodium sulfosuccinate, diamyl sodium sulfosuccinate, the lower concentrations of 2-amino-2-methyl-1,3-propanediol, or triethanolamine. Negative results are reported, however, because detectable separation occurred within the arbitrary 7-day period.

Microscopic examination of the products containing D & C red No. 18 dye, predissolved in the oil, confirmed the fact that emulsification had occurred. Figures 2 and 3 illustrate this observation. In all cases, except that of sodium stearate, the oil appeared in the internal phase, although evidence of

TABLE II.—EMULSIFYING EFFECTS OF CATIONIC SURFACTANTS ON EQUAL WEIGHTS OF GLYCERIN AND OLIVE OIL

Agent	%	Method I[a]	Method II[a]	Emulsion, Appearance
Benzalkonium chloride[b]	0.1	Separation	Separation	...
	0.2	Separation	Separation	...
	0.5	Separation	Separation	...
	1.0	Separation	Separation	...
	2.0	Separation	Separation	...
	5.0	Separation	Separation	...
	10.0	Separation	Separation	...
Lauryltrimethylammonium bromide[c]	0.1	Separation	[e]	...
	0.5	Partial sep.[d]	...	...
	1.0	Partial sep.[d]	...	...
	5.0	Partial sep.[d]	...	...
Cetylpyridinium chloride[c]	0.1	Separation	Separation	...
	0.5	Emulsification	Separation	Opaque
	1.0	Emulsification	Separation	Opaque
	5.0	Emulsification	Separation	Opaque
Stearyltrimethylammonium chloride[c]	0.1	Separation	Separation	...
	0.5	Partial sep.	Partial sep.	...
	1.0	Emulsification	Partial sep.	Opaque
	5.0	Emulsification	Emulsification	Opaque
Stearyldimethylbenzylammonium chloride[c]	0.1	Separation	Separation	...
	0.5	Emulsification	Separation	Opaque[f]
	1.0	Emulsification	Separation	Opaque[f]
	5.0	Emulsification	Separation	Opaque[f]

[a] See text. [b] Benzalkonium chloride (Zephiran Chloride, Winthrop Laboratories) was obtained by evaporation of a 12.8% aqueous solution and as the 92.7% semisolid. Both were employed in these experiments. The 92.7% semisolid produced emulsions in the higher concentrations, but separation occurred within 7 days. [c] Obtained from K & K Laboratories, Inc. [d] Emulsification was apparent by macroscopic and microscopic examination, but separation was detectable within 7 days. [e] Insoluble in the olive oil. [f] Opacity due to precipitated surfactant.

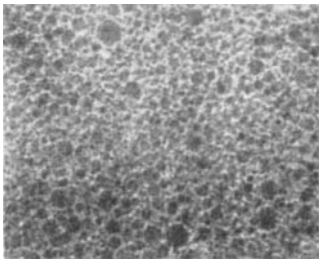

Fig. 2.—Photomicrograph of an emulsion containing 1.0% triethanolamine in equal weights of glycerin and olive oil stained with D & C red No. 18 dye.

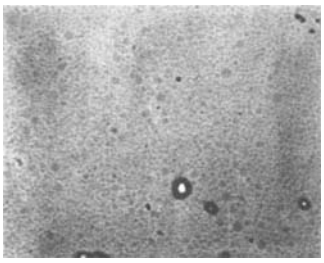

Fig. 3.—Photomicrograph of an emulsion containing 3% Aerosol in equal weights of glycerin and olive oil stained with D & C red No. 18 dye.

mixed emulsification was observed in a number of instances. Figure 4 is a photomicrograph of a sodium stearate emulsion wherein the oil (dark area) is seen in the external phase. Mixed emulsification can be detected by close observation of this figure.

When sodium lauryl sulfate, sodium stearate, or ethanolamine was used, the resulting preparations had a very thick consistency. Indeed, the higher concentrations resulted in semisolid preparations. Consequently, any separation in these products occurred very slowly, usually over several weeks.

With the exception of ethanolamine, the saponifying amines produced very stable emulsions, even in very low concentrations. The resulting products were clear or only faintly cloudy. Several of them exhibited opalescence. Some darkening was noted in emulsions containing triethanolamine or ammonia on standing. However, some of these preparations have remained stable for over 2 years. It is interesting that the use of preformed triethanolammonium oleate, prepared by reacting equivalent amounts of triethanolamine and oleic acid, aided by mild heating, produced the same results as did the corresponding amount of triethanolamine.

Table II reveals that stable emulsions were obtained from the use of the cationic agents, cetylpyridinium chloride, stearyltrimethylammonium chloride, and stearyldimethylbenzylammonium chloride. Emulsions were formed also when benzalkonium chloride or lauryltrimethylammonium bromide were used; however, these emulsions separated, partially or completely, within 7 days. That emulsification occurred in these preparations was confirmed by microscopic examination of the dyed emulsions.

An inversion in emulsion type was observed when stearyltrimethylammonium chloride was employed in concentrations of 0.5 and 1.0% by Method I. Preparations containing 0.5% of this agent formed oil-in-glycerin emulsions until the proportion of oil reached approximately 42% of the total composition, at which point an inversion to the glycerin-in-oil type occurred. Partial separation of this emulsion was detectable after standing for 7 days. Preparations containing 1.0% stearyltrimethylammonium chloride, prepared by Method I, formed oil-in-glycerin emulsions until the proportion of oil reached approximately 48%, at which point inversion occurred. No separation of these emulsions was detectable after 7 days. At the 5.0% level of surfactant inversion to the glycerin-in-oil type did not occur, even when the oil concentration was increased to approximately 78–80%. At this point the emulsions "cracked."

The use of stearyltrimethylammonium chloride by Method II produced emulsions of the glycerin-in-oil type at concentrations of 0.5, 1.0, and 5.0%, although at the two lower concentrations partial separation occurred on standing for 7 days.

A comparison of the results obtained with Method I and Method II reveals that the former was superior in its ability to produce stable emulsions with either anionic or cationic surfactants. Method II was successful only in the case of ammonia, in one preparation containing 1.0% triethanolamine which had been aged for several days (not included in Table I), and in preparations containing stearyltrimethylammonium chloride.

DISCUSSION

The data presented in Table I indicate that certain anionic agents are useful for emulsifying the glycerin-olive oil system employed in these studies. This interpretation is supported by macroscopic and microscopic examination and by testing the products for miscibility with added olive oil or glycerin. Except for sodium stearate emulsions, all of the emulsions prepared from anionic agents were of the oil-in-glycerin type. Emulsions containing sodium stearate were of the glycerin-in-oil type. The sodium stearate–induced emulsions exhibited a degree of mixed emulsification, as did a number of other emulsions. This can be observed in Fig. 4. The glycerin-in-oil nature of emulsions containing sodium stearate was further substantiated by the fact that only a limited quantity of glycerin could be added to these emulsions, whereas olive oil was readily miscible with them. In all other instances glycerin, but not olive oil, mixed readily with the emulsions.

Emulsions obtained with 2-amino-2-methyl-1,3-propanediol, tris(hydroxymethyl) aminomethane, triethanolamine, ethanolamine, or ammonia, using Method I, formed rapidly with a minimum of agitation. Evidently, saponification occurred readily. The great capacity of these agents to emulsify the oil is remarkable, since, in one case, as little as 0.01% of the saponifying amine was used to produce a stable emulsion. Still lower concentrations may be effective for this purpose.

The value of the cationic surfactants as emulsifying and solubilizing agents has not been unequivocally established. The solubilizing effects of dodecylamine hydrochloride (11) and cetylpyridinium chloride (12) in aqueous preparations have been reported. The results presented herein indicate that cetylpyridinium chloride, stearyltrimethylammonium chloride, and stearyltrimethylbenzylammonium chloride are effective agents for the emulsification of olive oil and glycerin under the conditions of this experiment and may be of value in this application. These observations should be extended before categorical statements can be made regarding

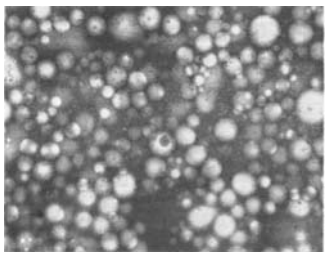

Fig. 4.—Photomicrograph of an emulsion containing 2.0% sodium stearate in equal weights of glycerin and olive oil stained with D & C red No. 18 dye.

the usefulness of these agents in solubilizing or emulsifying aqueous or nonaqueous systems.

The results reported in Table II on the cationic agents were substantiated by microscopic and macroscopic examination and by testing the products for stability and for miscibility with added olive oil or glycerin.

The fact that both types of emulsions, oil-in-glycerin and glycerin-in-oil, were induced by one agent, stearyltrimethylammonium chloride, lends support to the well known theories which imply that an emulsion forms in such a way that the higher proportion of surfactant is in the external phase. Indeed, with this agent, Method I (in which the surfactant was first dissolved in glycerin) favored the formation of oil-in-glycerin emulsification, whereas the reverse was true of Method II. The inversions observed during manipulation by Method I did not occur until substantial quantities of oil had been incorporated into the internal phase.

The viscosity of emulsions resulting from the use of either anionic or cationic agents was obviously much higher than that of either of the constituents, although accurate viscosity measurements have not yet been made. Even when emulsification was not effected, an increase in viscosity and slower rates of separation, compared to olive oil and glycerin alone, frequently were noted.

It was observed that in many instances where negative results are indicated, emulsification resulted until the proportion of oil exceeded a maximum concentration at which point separation occurred. This point was frequently very sharp, occurring on addition of only 1 or 2 ml. of oil and detectable by a sudden decrease in viscosity. This phenomenon was particularly noticeable with the lower concentrations of saponifying amines. For such cases it is assumed that the concentration in internal phase exceeded the capacity of the surfactant to maintain a stable emulsion. It is realized that greater variation in the concentrations of surfactants or in the proportion of glycerin and/or olive oil may have produced positive results where emulsification otherwise did not occur.

The addition of an oil soluble dye (D & C red No. 18) or a glycerin soluble dye (methylene blue) to the *finished emulsion* was unsatisfactory for microscopic observation and led to some early erroneous conclusions. The fact that most of the nondyed emulsions were translucent and failed to show emulsification on microscopic examination led to the initial belief that solubilization was the primary action of the surfactants in this system. This is understandable in view of the similar refractive indices of the reagents (glycerin n_D^{25} 1.4730; olive oil n_D^{25} 1.466–1.468). This also may account for the fact that most of the emulsions were clear or only faintly cloudy.

Technique played an important role in the successful formation of emulsions. Vigorous trituration and slow addition of the oil enhanced the chances of effecting emulsification. Microscopic examination of the dyed emulsions revealed that those prepared by this technique exhibited smaller globule sizes than those prepared by less vigorous agitation and more rapid addition of the oil. It should be mentioned, however, that the surfactant also had a pronounced influence on the size of the globules contained in the emulsion.

Why is Method I superior to Method II in its capacity to produce stable emulsions? The answer to this question is not obvious. However, based on the assumption that micelle formation is responsible for eventual emulsification, an explanation might be proposed. McBain (13) has suggested that emulsification results when the saturation point of micelles is exceeded and further states: "The apparent density of liquid solubilized by a micelle is the same or nearly the same as that of the pure oil at the limit of saturation (as may easily be seen on reflecting that when the micelle becomes saturated the addition of still more liquid results in a *transition from solution to emulsion*)." The existence of micelles in nonaqueous systems has been reported by several investigators (14–20). Hall (21), on the other hand, has evidence that micelles are not present in benzene solutions of the nonionic agent, polyoxyethylene lauryl ether 30,[1] but has evidence supporting the existence of micelles in benzene solutions of anionic and cationic surfactants. The fact that glycerin is a polar substance and has solvent properties quite similar to those of water would lead one to suspect that micelles form in glycerin in a manner similar to the micelle formation in water. The assumption that micelles do exist and that the oil is dissolved within the hydrocarbon interior of the micelle appears logical. The fact that emulsions formed quickly and easily by use of the saponifying amines indicates that micellar formation was rapid. It would further appear that once a micelle forms, the orientation is highly stable and a reversion in type does not readily occur. Thus, the micellar orientation within the system employed in these studies which leads to stable emulsions must, in most instances, be that present in glycerin.

SUMMARY

The effects of various surfactants on the system, olive oil and glycerin, were studied. The anionic agents, dioctyl sodium sulfosuccinate, diamyl sodium sulfosuccinate, and calcium stearate failed to produce stable emulsions under the conditions of this study. The cationic agents, benzalkonium chloride and lauryltrimethylammonium bromide, likewise failed to stabilize the system.

Some anionic agents were effective in producing emulsification of glycerin, and olive oil. Tetrasodium - N - (1,2 - dicarboxyethyl) - N - octadecylsulfosuccinamate, sodium lauryl ether sulfate, and sodium lauryl sulfate produced stable, somewhat opaque, preparations. The amines, 2-amino-2-methyl-1,3-propanediol, tris(hydroxymethyl) amino-

[1] Marketed as Brij 30 by the Atlas Powder Co., Wilmington, Del.

methane, ethanolamine, triethanolamine, and ammonia gas, at very low concentrations, formed saponification products which resulted in clear, stable emulsions. Three cationic agents, cetylpyridinium chloride, stearyltrimethylammonium chloride, and stearyldimethylbenzylammonium chloride, likewise produced stable emulsions of this system.

Method I, in which the surfactant was added to the glycerin, was effective in all of the cases mentioned; whereas Method II, in which the surfactant was added to the oil, was effective in only a few isolated cases—namely, with ammonia, triethanolamine, and stearyltrimethylammonium chloride. All of the emulsions formed were of the oil-in-glycerin type, with the exception of emulsions containing sodium stearate or stearyltrimethylammonium chloride.

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Comparative Activities of Maleyl, Fumaryl, and Succinyl Dicholine

A Correction of the Literature

By JOHN F. McCARTHY, JOSEPH G. CANNON†, and JOSEPH P. BUCKLEY

A reinvestigation of the synthesis and structure of "dicholine maleate" reported by an earlier worker has shown that the *cis*-double bond was isomerized to the *trans*-form. An alternate synthesis for dicholine maleate has been repeated and has been found to give rise to the correct structure. Contrary to literature reports, there are marked differences in biological potency between maleyl and fumaryl dicholines compared with succinyl dicholine.

In considering structure-activity relationships of succinyl dicholine, conformations of the succinic acid portion of the ester assume some importance. If one considers possible conformations of a succinate ester, it would be expected that a staggered conformation of the acid would be favored over eclipsed or gauche forms. Inspection of Dreiding models of maleic and fumaric acids reveals that the carboxyl groups of these acids are oriented in space in a manner which closely resembles the orientation in space of the eclipsed and the staggered conformations, respectively, of succinic acid. The question then arises as to whether the staggered conformation would be favored for adsorption at a biological receptor surface.

Dicholine esters of maleic and fumaric acids offer special interest in that they are unsaturated analogs of succinyl dicholine. The double bond confers some degree of rigidity upon the acid portion of the system; thus the quaternary groups are held in a somewhat fixed, rigid relationship to each other, being relatively close together in the *cis*-maleate ester, and relatively far apart in the *trans*-fumarate ester. If the concept that both quaternary heads of succinyl dicholine attach in some specific manner to receptor sites and/or to "anchoring" sites is valid, there should be considerable difference in biological potency between the dicholine esters of the two isomeric unsaturated acids. Cavallito and Gray (1) stated that the fumaryl and the maleyl esters of choline

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methane, ethanolamine, triethanolamine, and ammonia gas, at very low concentrations, formed saponification products which resulted in clear, stable emulsions. Three cationic agents, cetylpyridinium chloride, stearyltrimethylammonium chloride, and stearyldimethylbenzylammonium chloride, likewise produced stable emulsions of this system.

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are equally and fairly active. Brücke (2) cited literature reports that fumaryl dicholine is approximately one-half as potent as succinyl dicholine in the rabbit head-drop test. This author, while listing maleyl dicholine in his tables, recorded no biological data for it. Glick (3) reported synthesis of maleyl dicholine by condensing maleic anhydride with ethylene bromohydrin using heat but no solvent or catalyst, followed by treatment of the resulting bis-(2-bromoethyl) maleate with an excess of trimethylamine. Glick did not report test data of succinyl dicholine-like activity for this ester; hydrolysis rates for the compound which were reported by Glick were extremely similar to those reported by other workers for analogous fumarate esters. Synerholm and Hartzell (4) reported the preparation of bis-(2-chloroethyl) maleate by reacting 2-chloroethanol with maleic anhydride. When the experiments of Glick and of Synerholm and Hartzell leading to bis-(2-haloethyl) maleates were repeated in this laboratory, products were obtained which corresponded in melting points and in other physical characteristics to those reported by the original workers; however, these products were identical (evidenced by infrared spectral studies and by mixed melting points) to fumarate esters which were prepared in an unumbiguous manner. Therefore, it would appear that the hydrogen halide, formed in small amounts during the reaction of maleic anhydride with the halohydrin, catalyzed isomerization of the *cis* double bond to the more stable *trans*. It is well established (5) that maleates are rapidly converted to fumarates in the presence of hydrohalic acids. It would appear that Glick's "maleyl dicholine" was actually fumaryl dicholine.

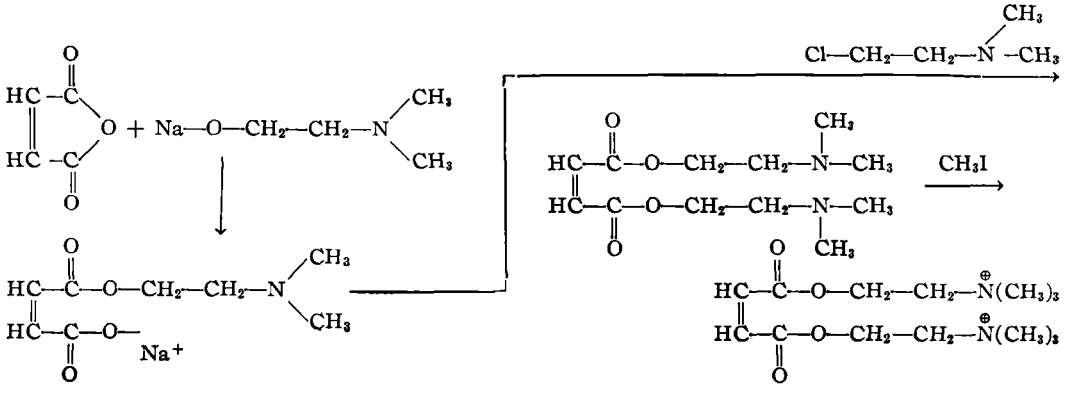
The only other recorded synthesis of maleyl dicholine is that of Christiansen and co-workers (6), who reported no data to support the structure of the material claimed to be maleyl dicholine. Hence, their synthesis was repeated in this laboratory as outlined

Mayo and Walling (7) have stated that primary and secondary amines will cause isomerization of maleates to fumarates. However, these authors pointed out that tertiary amines (except for pyridine) are known not to effect this isomerization. Hence, it was expected that no change in the configuration about the double bond would occur in the preparation of the bis-(2-dimethylaminoethyl) ester of maleic acid. Confirmation of this was obtained when the infrared spectrum of bis-(2-dimethylaminoethyl) fumarate (prepared from bis-(2-bromoethyl) fumarate and dimethylamine) was found to be significantly different from that of bis-(2-dimethylaminoethyl) maleate prepared by the method of Christiansen and co-workers (6).

Application of classical methods to prove that treatment of bis-(2-dimethylaminoethyl) maleate with excess methyl iodide did not result in isomerization of the double bond and that the final product of the reaction sequence was indeed dicholine maleate led to inconclusive results. When dicholine maleate, prepared in this laboratory by the method of Christiansen and co-workers (6), was hydrolyzed with base, only fumaric acid could be isolated following acidification. This result is not necessarily significant, since the iodide ions present in the solution will be converted into hydriodic acid on acidification of the reaction mixture with sulfuric acid. In addition, air oxidation could form small amounts of free iodine which is known (5) rapidly to isomerize maleic to fumaric acid. The fact that the acidic solution from which the fumaric acid was isolated assumed the color of a dilute solution of iodine supports this contention.

A comparison of the decomposition points of an authentic sample of dicholine fumarate and of dicholine maleate prepared by Christiansen's method revealed that the two products melted with decomposition at the same temperature (252–253°) when determined under identical conditions, individually, and when mixed. Generally, these data are taken as proof that two samples from different sources are identical. However, it has been reported (8) that maleic acid is converted to the higher melting fumaric acid when it is heated slightly above its melting point; hence, it could be argued that at such high temperatures, dicholine maleate might be expected to isomerize to dicholine fumarate.

Infrared spectroscopy would not be expected to furnish conclusive results; the dicholine esters are completely insoluble in solvents used for infrared studies. Hence, it would be necessary to determine



their spectra in the solid state. Differences in spectra of closely related compounds taken in the solid state can be due to differences in crystalline forms rather than to differences in molecular structure. Stafford and his co-workers (9) have stated that the absorption maxima of maleates and of fumarates in the ultraviolet occur at the same wavelength (210 mμ region), but that the extinction coefficient of fumarates is greater than that of maleates. When the ultraviolet spectra of dicholine maleate diiodide and of dicholine fumarate diiodide were recorded in methanol, an absorption maximum appeared at 219 mμ, with an extinction coefficient of 15,000 in both cases. This maximum is probably not due to the unsaturated chromophore but to the iodide ion. Knight and co-workers (10) have reported that the ultraviolet spectrum of tetramethylammonium iodide contains an absorption maximum at 226 mμ with an extinction coefficient of 13,300. These workers attributed this absorption to iodide ion.

Support for the structure of dicholine maleate was obtained by paper chromatographic analysis (see Table I). Goldenberg and Spoerri (11) found that esters of dicarboxylic acids in which the two ester groups were in close proximity, such as diesters of maleic and phthalic acids, reacted more slowly with aqueous alkaline hydroxylamine to give hydroxamic acids than did diesters of fumaric acid or diesters in which the ester groups were separated by more than two carbons. Also, a much less intense color was produced in the case of maleic and phthalic acids on addition of ferric chloride reagent. The fact that the di-quaternary compound arising from bis-(2-N,N-dimethylaminoethyl) maleate failed to develop a color when paper chromatograms were treated with the hydroxylamine–ferric chloride reagent was interpreted as evidence that no isomerization of the double bond had occurred.

The differences which were noted in biological activities of the dicholine esters of maleic and fumaric acids further substantiated the structure of the dicholine maleate prepared by the method of Christiansen and co-workers.

Schilling and Pedersen (14) reported that maleyl dicholine prepared by Christiansen and co-workers (6) had some ability to paralyze skeletal muscle. However, these workers did not compare similar dose levels of maleyl dicholine and of succinyl dicholine, and they did not report tests on fumaryl dicholine. A search of the literature has not revealed that any group has prepared authentic maleyl dicholine and carried out parallel tests on it, on fumaryl dicholine, and on succinyl dicholine. The importance of steric factors in structure-activity relationships of quaternary compounds indicates the desirability of concurrent tests of these three esters.

TABLE I.—PAPER CHROMATOGRAPHIC ANALYSIS OF DICHOLINE MALEATE AND OF DICHOLINE FUMARATE[a]

Dicholine Ester	Reaction to Iodine (12)	Reaction to Hydroxylamine-ferric chloride (13)	R_f Value
Maleate	+	−	0.18
Fumarate	+	+	0.18

[a] The paper chromatographic system used was that of Augustinsson, K. B., and Grahn, M., *Acta Chem. Scand.*, 7, 906(1953).

The threshold dose of succinyl dicholine, determined in 13 cats, was approximately 0.04 to 0.05 mg./Kg., with a duration of action between 2 and 14 minutes.

Fumaryl dicholine produced a blockade of skeletal muscle contraction at a threshold dose of approximately 0.12 mg./Kg. Doses ranging between 0.06 and 2.32 mg./Kg. were administered to four cats. The compound did not produce significant changes in blood pressure below this threshold dose; however, doses above 0.2 mg./Kg. produced pressor effects and marked respiratory depression.

Maleyl dicholine was much less effective than either fumaryl dicholine or succinyl dicholine, and the threshold dose was approximately 2.0 mg./Kg. Doses ranging between 0.1 and 4.38 mg./Kg. were administered to four cats. The compound also produced transient hypotensive effects in the lower dose range (0.1 to 2.0 mg./Kg.) and marked hypotensive effects in doses in excess of 2.0 mg./Kg.

EXPERIMENTAL[1]

Chemical

Bis-(2-bromoethyl) Fumarate.—*A. From Fumary Chloride.*—Sodium dried, thiophene-free benzene (125 ml.), 17 Gm. (0.175 mole) of anhydrous reagent grade potassium carbonate, and 20 ml. (34.5 Gm., 0.276 mole) of 2-bromoethanol were mixed in a 500-ml. three-neck flask equipped with a mechanical stirrer, dropping funnel, and a condenser topped with a drying tube. A 14.5-Gm. (0.095 mole) quantity of fumaryl chloride (15) in 125 ml. of benzene was added over a 15-minute period to the rapidly stirred contents of the flask. Stirring was continued for 3 to 4 hours; then 100 ml. of chloroform was added, and the insoluble inorganic material was removed by filtration. The filtrate was washed successively with 100 ml. of water, two 100-ml. portions of saturated sodium bicarbonate solution, and 100 ml. of water. Removal of the solvent under reduced pressure (after drying with sodium sulfate) gave 22.1 Gm. (70%) of a white solid, which was recrystallized twice from Skelly B; m.p. 66–67°.

Anal.—Calcd. for $C_8H_{10}Br_2O_4$: C, 29.12; H, 3.05; Br, 48.44. Found: C, 29.76; H, 3.17; Br, 47.45.

B. From Maleic Anhydride.—A 10.65-Gm. (0.11 mole) quantity of freshly sublimed maleic anhydride (or Eastman white label grade, without sublimation) and 14.3 ml. (25 Gm., 0.2 mole) of 2-bromoethanol were placed in a 250-ml. round bottom flask equipped with a condenser and drying tube. The mixture was heated at 100° for 9 hours, cooled, and 200 ml. of chloroform was added. The insoluble material (4.8 Gm.) was removed by filtration; the filtrate was washed successively with 50 ml. of water, two 50-ml. portions of saturated sodium bicarbonate solution, and two 50-ml. portions of water. The chloroform solution was dried over sodium sulfate; the solvent was removed on a steam bath giving 14.5 Gm. (40%) of a solid which was recrystallized from Skelly B; m.p. 65–66°. [Glick (3) reported m.p. 66° for the neutral product of this reaction. Synerholm and Hartzell (4) reported m.p. 66.5 to 68°.] The infrared spectrum (8% in chloroform) was identical

[1] All melting points are uncorrected. Elemental analyses were performed by Huffman Microanalytical Laboratories, Wheatridge, Colo.

in all respects with that of the product from *A* (8% in chloroform). Also, a mixed melting point determination with the product from *A* showed no depression.

Bis-(2-N,N-dimethylaminoethyl) Maleate.—This compound was prepared in 4% yield by the method of Christiansen and co-workers (6); b.p. 109–110° (0.1 mm.); literature (6) b.p. 145° (1.5 mm.).

Bis-(2-N,N-dimethylaminoethyl) Fumarate.—A 6.0-Gm. (0.018 mole) quantity of bis-(2-bromoethyl) fumarate and 3.6 Gm. (0.08 mole) of dimethylamine were added to 100 ml. of sodium-dried toluene in a 125-ml. Erlenmeyer flask fitted with a ground-glass stopper. The flask was stoppered and allowed to stand at room temperature for 1 month. The insoluble material was removed by filtration, the toluene was removed from the filtrate under reduced pressure, and the residue was distilled, whereupon 4.3 Gm. (90%) of material boiling at 105–106° (0.02 mm.) was collected; literature (16) b.p. 138° (2 mm.).

Dicholine Maleate and Dicholine Fumarate.—These compounds were prepared by the procedure described by Christiansen and co-workers (6) for the preparation of methiodides. Reagent grade acetone was employed; it was stored over anhydrous potassium carbonate. The methyl iodide was Eastman white label grade, and it was redistilled immediately before use. Both dicholine ester diiodides were recrystallized from methanol-water (5:1). Melting point determinations were performed in an open capillary tube which was placed in a rapidly stirred bath preheated to 230°, and the temperature was increased at the rate of 2° per minute. The melting points of the two esters were determined simultaneously, and they were both 252–253° with decomposition. A mixture of equal parts of the two esters melted at 252–253° with decomposition under conditions described above. Bovet and co-workers (16) reported 253° for dicholine fumarate and Christiansen and co-workers (6) reported 264° for dicholine maleate.

Pharmacology

Cats of each sex were anesthetized with α-chloralose and urethan (30 mg./Kg. in a 25% urethan solution). The dose utilized was calculated on α-chloralose as 55 mg./Kg.

After cannulating the trachea, the ipsilateral femoral nerve was severed. The posterior tibial nerve was separated from the peroneal nerve for a distance of 2 or 3 cm. The tibial nerve was ligated at a distance of 4 to 5 cm. from its union with the peroneal nerve and cut distal to the ligature. If bleeding occurred from the small vessels in the neural sheath, a piece of surgical gauze soaked in thrombin (100 NIH units/ml.) was applied to the nerve to aid in coagulation. The bony tubercle on the medial edge of the foot between the heel and toe was detached with bone clippers and the tendon of the tibialis anticus muscle ligated just above the tubercle with a strong thread. The distal end of the thread was attached to a muscle pulley lever system. The recordings of the contractions of the tibialis anticus muscle were made on a slowly moving smoked kymograph. Platinum tipped electrodes were fixed around the intact peroneal nerve. The nerve was bathed in warm heavy mineral oil maintained at 35 to 38°. A Grass stimulator (SD5) was used to deliver 0.8 to 1.0 volt square wave impulses every 6 seconds. The threshold for the peroneal nerve was determined for each preparation and control contractions recorded for a 30-minute period. A carotid artery was cannulated, and the blood pressure was recorded by means of a glass cannula and mercury manometer system. The glass cannula was connected to its respective pressure recording device through a normal saline (0.1% sodium chloride solution) bridge. Coagulation was prevented by injecting 0.2 ml. of heparin (1000 U.S.P. units/ml.) into the tip of the glass cannula. The experimental compound was administered *via* the femoral vein and the effects on contraction of the tibialis anticus muscle and blood pressure recorded. Succinyl dicholine was administered to each preparation.

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Chemical and Pharmacological Studies on *Argemone mexicana*

By B. C. BOSE, R. VIJAYVARGIYA, A. Q. SAIFI, and S. K. SHARMA

Chemical and pharmacological studies of Argemone mexicana have been carried out. Chemical analysis revealed the presence of total alkaloids 0.125 per cent, consisting of protopine 0.084 per cent and berberine 0.041 per cent, tannin 1.10 per cent, resin 1.75 per cent, and a toxic principle in argemone oil. The main activity was related to the alkaloids, the protopine fraction stimulating heart, respiration, skeletal muscles, and blood pressure. The total alkaloidal fraction stimulated all the smooth muscles and antagonized the actions of acetylcholine, histamine, and 5-hydroxytryptamine. Its oxytocic action resembled that of pitocin. Atropine, adrenergic blocking agents, and antihistaminics did not modify the response on blood pressure. The carotid occlusion reflex was inhibited, but responses to acetylcholine and catecholamines remained unaffected. It antagonized barbiturate-induced depression and potentiated methamphetamine stimulation of spontaneous motility in mice. It produced a mild neuromuscular block of the diaphragm and showed antiacetylcholine action on the frog rectus.

ARGEMONE MEXICANA (*Papaveraceae*), an American plant naturalized in India, grows widely and is commonly known as a pivla dhatura or satyanashi. The yellow, milky juice has long been used in indigenous systems of medicine for dropsy, jaundice, skin diseases, indolent or syphilitic ulcers, eye conditions, respiratory disorders, and constipation. Schlotterbeck (1) isolated and identified two alkaloids, berberine and protopine, from the plant. Berberine was tried by the early physicians in malaria and leishmaniasis (2–4). The seeds of this plant yield a fixed oil, argemone oil (22 to 36%), which, when consumed in adulterated mustard oil for cooking, was found responsible for the epidemic dropsy in certain areas (2). Mukerji (5) isolated a toxic substance, with an empirical formula $C_{19}H_{15}NO_4$, responsible for this. Pharmacological actions of berberine, obtained from the berberis family, have been reported (6, 7). Since no investigations on the effects of total alkaloids, and especially the protopine fraction of this plant have so far been reported, the present study was undertaken.

EXPERIMENTAL

The air-dried powdered roots and stems of the plant were subjected to successive extractions with the following solvents in a soxhlet apparatus and the residues found to be: alcohol—10.5, chloroform—2.1, petroleum ether—2.7, benzene—0.9, ether—1.7, acetone—3.3, and ethylacetate—1.6%.

On incineration, the total, acid soluble, acid insoluble, and water soluble ashes were found to be 8.56, 85.3, 14.7, and 50.8%, respectively. Inorganic constituents detected were potassium, calcium, sodium, aluminum, sulfates, nitrates, and carbonates.

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The paper chromatographic studies of alcohol and chloroform extracts, using Whatman No. 1 filter paper and water-saturated *n*-butanol:glacial acetic acid (25:1) as solvent, gave two distinct spots with R_f values of 0.35 and 0.23 and golden yellow and bluish fluorescence, respectively. The chromatograms, when sprayed with modified Dragendorff's reagent, showed them to be due to alkaloids. These were found to agree with berberine and protopine, respectively.

The plant was also found to contain tannins (1.10%) and resin (1.75%) by potassium permanganate titration and water precipitation methods, respectively. All the extracts gave a positive Libermann-Burchard's test for sterol bodies which was later found to be due to the alkaloids themselves.

The alkaloids were quantitatively estimated by the method described by Alfredo Santos (8). Much variation was observed from sample to sample, but the average total alkaloidal content was 0.125%. Of these, protopine formed 0.084%. The presence of these alkaloids was confirmed by the melting points and maximum light absorption spectra.

The toxic principle from the seed was separated and identified from the oil according to the method described by Mukerji (5).

Most of the pharmacological experiments were conducted with a total alkaloidal solution with the hydrochloride salt. In a few cases, both berberine and protopine were used for comparative evaluation of differences in actions.

Ten dogs, (five anaesthetized with urethane, 1.8 Gm./Kg., and five with diallyl barbituric acid, 0.7 ml./Kg.), weighing between 6–13 Kg., were utilized in the present study for observations on the effects of alkaloids on blood pressure, respiration, auriculo-ventriculogram, and intestine by the usual techniques.

Blood Pressure.—The total alkaloidal fraction was studied in a dose range of 0.05 to 5.0 mg./Kg. Smaller doses either had no effect or occasionally produced a slight rise, but a dose above 0.1 mg./Kg. produced a definite fall in blood pressure (Fig. 1). Atropine, adrenergic blocking agents, or antihistaminics did not modify the response. The hypotensive effect was more marked in experimentally induced hypertension with norepinephrine.

The sinus response due to carotid occlusion was inhibited by this fraction, but responses due to acetylcholine, epinephrine, or norepinephrine remained unaltered (Fig. 2). With smaller concentrations, a potentiation of acetylcholine-induced fall in blood pressure was observed.

Protopine fraction produced a rise in the blood pressure in smaller concentrations and a fall in doses greater than 1 mg./Kg. Berberine produced a fall in all the concentrations tried.

Heart.—The total alkaloidal fraction (1.0 mg./Kg.) inhibited both auricles and ventricles *in situ* in dogs. Rabbit heart, both *in situ* and isolated, was also depressed by the alkaloids in a concentration of 1×10^{-5} to 4×10^{-4}. Isolated amphibian heart was depressed even in a concentration of 1×10^{-6}. It also antagonized acetylcholine and epinephrine responses in a concentration of 1×10^{-5} to 2×10^{-4} (Fig. 3). Whereas the protopine fraction produced a slight stimulation even in a dose of 25 mcg./Kg., the berberine fraction was consistently found to be a myocardial depressant.

Respiration.—The total alkaloidal fraction, when given intravenously, stimulated respiration. It antagonized barbiturate-induced respiratory depression at higher concentrations (Fig. 4). The protopine fraction was very potent in this respect, whereas berberine produced an initial stimulation followed by depression. Both atropine and dihydroergotamine did not affect these actions.

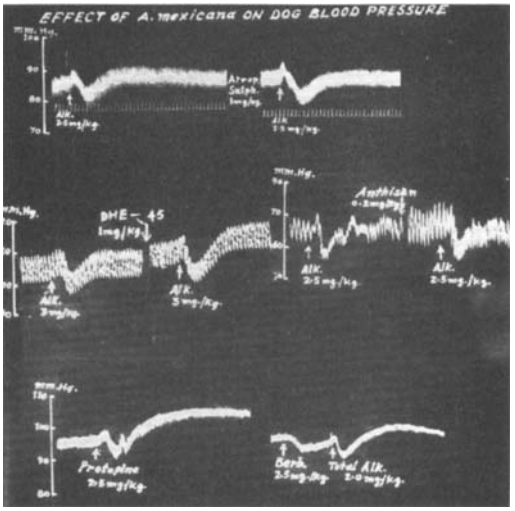

Fig. 1.—Effect of total alkaloid, protopine and berberine on blood pressure in the dog.

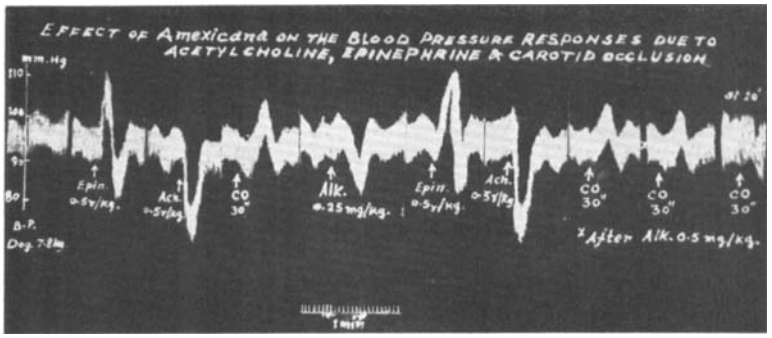

Fig. 2.—Effect of *A. mexicana* on the blood pressure responses to acetylcholine, epinephrine, and carotid occlusion responses.

Intestine.—In anesthetized dog and rabbit, both total and individual alkaloidal fractions produced an immediate stimulation of tone and peristaltic movements of the gut in a dose of 0.05 mg./Kg. Atropine inhibited the increase in tone but did not affect the augmentory action of the alkaloid on peristalsis.

Segments of ileum and uterus from freshly killed rats, guinea pigs, and rabbits, were mounted in an isolated organ bath and the effect of the alkaloids studied by the usual method. The intestine was stimulated in a concentration of 1×10^{-7} to 2×10^{-6}, with a concentration of 5×10^{-5}, though tone was increased; but there was a decrease in the amplitude of rhythmic contractions. In a concentration of 2×10^{-6} to 1×10^{-5} the total alkaloidal fraction administered half a minute before or simultaneously with spasmogens produced antiacetylcholine, antihistamine, and anti-5-hydroxytryptamine actions, even though it produced stimulation of its own. But when these spasmogens were allowed to remain in contact for some time and a persistent increased tone level obtained, the alkaloid produced an immediate relaxation. In atropinized tissue preparations the alkaloid still produced stimulation, though not markedly.

Other Smooth Muscles.—*In situ* experiments with rabbit uterii and isolated rat and guinea pig uterii showed that the alkaloid possessed a significant oxytocic activity in a concentration exceeding 5×10^{-7}; concentration of 5×10^{-6} produced, qualitatively, an effect similar to that of pitocin, 0.05 units/ml. (Fig. 5).

With frog blood vessel perfusion experiments, it was observed that the alkaloid produced vasodilatation in a concentration of 1×10^{-7} to 6×10^{-5}.

The effect on bronchioles was studied by both tracheal chain and lung perfusion experiments by the techniques detailed earlier (9). It was observed that though the total alkaloidal fraction produced a bronchoconstriction it exhibited antiacetylcholine and antihistaminic actions in a concentration of 5×10^{-6} to 3×10^{-4}.

Skeletal Muscle.—The protopine fraction, in a dose of 2.5 mg./Kg. or above, produced generalized muscle twitchings, more predominant in the neck and limb muscles, lasting for 2–5 minutes in dogs. This response usually corresponded with the respiratory stimulation. In a concentration of 2×10^{-5} to 2×10^{-4}, the total alkaloidal fraction showed an antiacetylcholine activity on frog rectus abdominis muscle studied in a leech muscle apparatus. A concentration of 2×10^{-4} evoked muscle twitchings. With experiments on a rat phrenic nerve diaphragm preparation by the method of

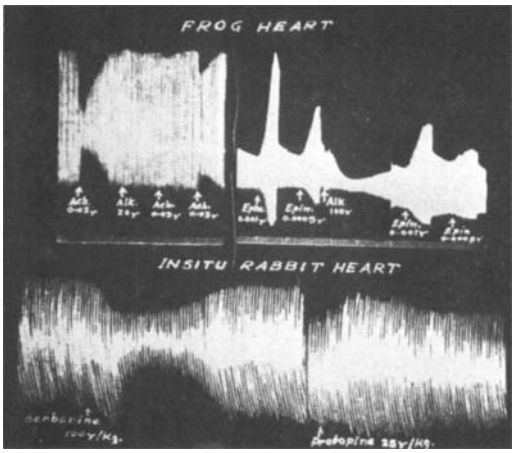

Fig. 3.—Effect of *A. mexicana* on frog and rabbit hearts.

Bulbring (10) it was observed that total alkaloidal fraction, in a concentration of 2×10^{-4}, produced a mild neuromuscular blocking effect which was partially antagonized by prostigmine. Concentrations of 2×10^{-5} or above produced muscle twitchings.

Effect of the total alkaloidal fraction on spontaneous motility was studied in mice (in groups of two each) with a simple instrument. It antagonized barbiturate-induced narcosis and potentiated the methamphetamine-induced hypermotility. The total alkaloidal fraction had no effect on rabbit cornea, conjunctival reflexes, or at the site of local injection in rats. But crude chloroform and petroleum ether extracts, suspended in normal saline, produced edema and necrosis at the site of injection in 80% of the cases within 2–3 days.

A few experiments conducted with the toxic substance isolated from argemone oil, showed hypotension, myocardial, respiratory, and intestinal inhibition. There was always a local action at the site of parenteral administration in rats.

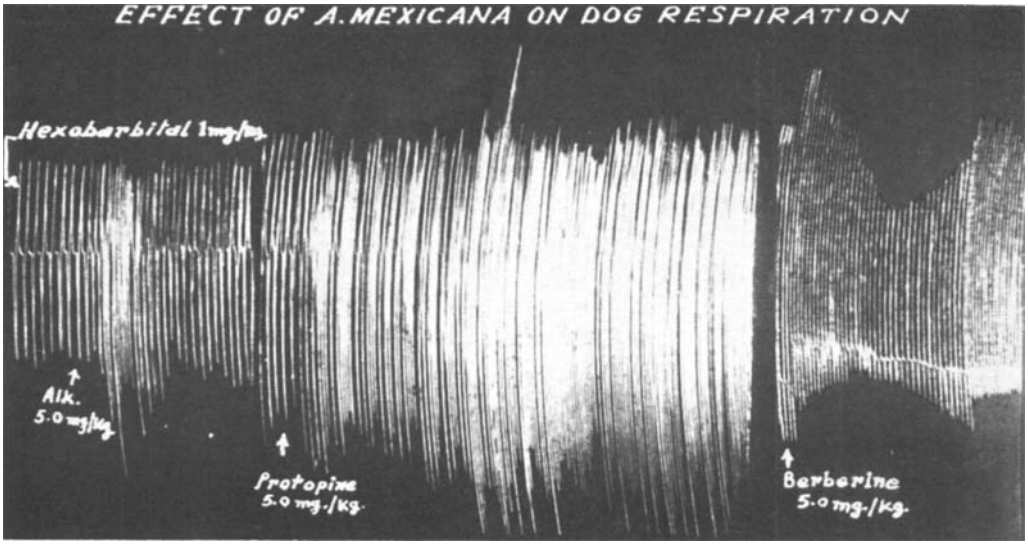

Fig. 4.—Effect of protopine and berberine on hexobarbital-depressed respiration of dog.

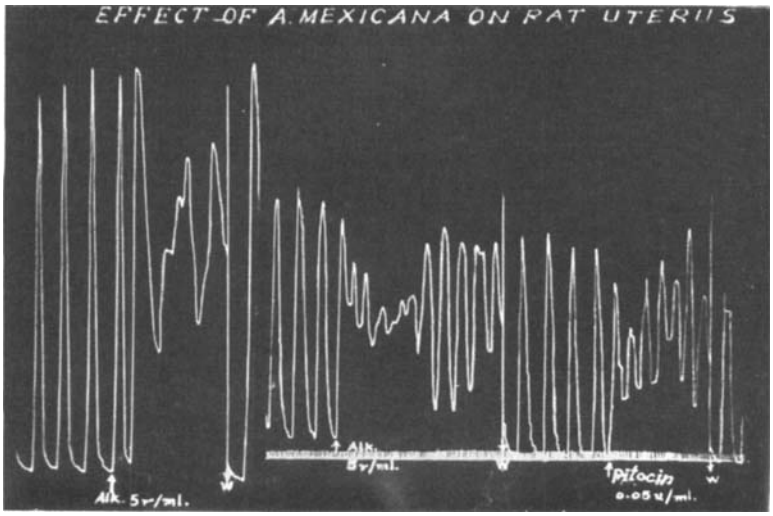

Fig. 5.—Effect of total alkaloid on isolated rat uterus.

Our findings with berberine on the gastrointestinal, cardiovascular, and respiratory systems are in agreement with those of other workers (6).

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Behavior of Erythrocytes in Various Solvent Systems I

Water-Glycerin and Water-Propylene Glycol

By DONALD E. CADWALLADER

The hemolytic behavior of rabbit and human erythrocytes in water–glycerin and water–propylene glycol solutions was investigated. Complete hemolysis of erythrocytes took place in all propylene glycol solutions and most glycerin solutions. Aqueous solutions containing 50, 60, and 70 per cent glycerin prevented complete hemolysis of rabbit erythrocytes but not human erythrocytes. The addition of sodium chloride to various glycerin solutions prevented hemolysis. The addition of sodium chloride to propylene glycol solutions prevented hemolysis of rabbit erythrocytes in 5–30 per cent solutions and of human erythrocytes in 5–40 per cent solutions. When possible, the data were used to calculate van't Hoff *i* values for sodium chloride in various water-glycerin and water–propylene glycol solutions. Unusual behavior was displayed by erythrocytes in 40–50 per cent propylene glycol solutions. The addition of sodium chloride to solutions containing 50 per cent or more of propylene glycol did not prevent complete laking of red blood cells.

SINCE THE DEVELOPMENT of the hemolytic method by Husa and co-workers (1, 2), many investigations have been carried out (3–12) to study the behavior of erythrocytes to various compounds. In the aforementioned investigations, water was used as the solvent for all of the substances studied. Water, however, is not the only solvent used for intravenous preparations. To prepare a safe, stable, and efficacious injection, it is sometimes necessary to employ a mixed solvent system consisting of water and a nonaqueous co-solvent. Two nonaqueous solvents that are used in the formulation of parenteral preparations are glycerin and propylene glycol.

Husa and Adams (1) showed that glycerin and propylene glycol did not prevent hemolysis at concentrations which were calculated to be isotonic according to physicochemical data. They also reported that 0.3 to 0.5% sodium chloride would prevent hemolysis when added to hypo-osmotic concentrations of the polyhydric alcohols in water. Hammarlund and Pedersen-Bjergaard (13) demonstrated that complete hemolysis of blood takes place in iso-osmotic concentrations of glycerin and propylene glycol.

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Our findings with berberine on the gastrointestinal, cardiovascular, and respiratory systems are in agreement with those of other workers (6).

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Behavior of Erythrocytes in Various Solvent Systems I

Water-Glycerin and Water-Propylene Glycol

By DONALD E. CADWALLADER

The hemolytic behavior of rabbit and human erythrocytes in water–glycerin and water–propylene glycol solutions was investigated. Complete hemolysis of erythrocytes took place in all propylene glycol solutions and most glycerin solutions. Aqueous solutions containing 50, 60, and 70 per cent glycerin prevented complete hemolysis of rabbit erythrocytes but not human erythrocytes. The addition of sodium chloride to various glycerin solutions prevented hemolysis. The addition of sodium chloride to propylene glycol solutions prevented hemolysis of rabbit erythrocytes in 5–30 per cent solutions and of human erythrocytes in 5–40 per cent solutions. When possible, the data were used to calculate van't Hoff *i* values for sodium chloride in various water-glycerin and water–propylene glycol solutions. Unusual behavior was displayed by erythrocytes in 40–50 per cent propylene glycol solutions. The addition of sodium chloride to solutions containing 50 per cent or more of propylene glycol did not prevent complete laking of red blood cells.

SINCE THE DEVELOPMENT of the hemolytic method by Husa and co-workers (1, 2), many investigations have been carried out (3–12) to study the behavior of erythrocytes to various compounds. In the aforementioned investigations, water was used as the solvent for all of the substances studied. Water, however, is not the only solvent used for intravenous preparations. To prepare a safe, stable, and efficacious injection, it is sometimes necessary to employ a mixed solvent system consisting of water and a nonaqueous co-solvent. Two nonaqueous solvents that are used in the formulation of parenteral preparations are glycerin and propylene glycol.

Husa and Adams (1) showed that glycerin and propylene glycol did not prevent hemolysis at concentrations which were calculated to be isotonic according to physicochemical data. They also reported that 0.3 to 0.5% sodium chloride would prevent hemolysis when added to hypo-osmotic concentrations of the polyhydric alcohols in water. Hammarlund and Pedersen-Bjergaard (13) demonstrated that complete hemolysis of blood takes place in iso-osmotic concentrations of glycerin and propylene glycol.

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data it was possible to calculate *hemolytic* isotonic coefficients for sodium chloride in various water–polyhydric alcohol solutions (*e.g.*, *i* values for NaCl in 5, 10, 20% propylene glycol).

EXPERIMENTAL

Materials.—Propylene glycol U.S.P. and reagent grade glycerin and sodium chloride were used.

Preparation of Solutions.—All of the polyhydric alcohol solutions were weight-in-volume percentage preparations. The sodium chloride solutions were prepared in the same manner described by other workers (1–10).

Collection of Blood.—The blood was collected and defibrinated in the same manner reported by Husa and co-workers (1–10). The human blood used in these experiments was obtained from the forearm veins of a 31-year-old white male.

Quantitative Determination of Per Cent Hemolysis.—The method used to determine the degree of hemolysis of erythrocytes in the various solutions involved in this investigation was essentially that described by Grosicki and Husa (2). The laking of blood in distilled water was used as the standard for 100% hemolysis. Because of the viscosity of the polyhydric alcohol solutions, especially in high concentrations, it was necessary to use centrifuge speeds of 2000–3000 r.p.m. to bring about complete settling of intact cells.

Water-glycerin and water–propylene glycol solutions absorbed a small amount of light, and this absorbance increased with an increase in polyhydric alcohol content. This absorbance was determined for the various concentrations of polyhydric alcohols used in experiments, and these blank readings were subtracted from the Klett-Summerson colorimeter readings obtained at the end of hemolysis experiments.

After several experiments, complete hemolysis of blood in polyhydric alcohol solutions gave absorbance readings indicating greater than 100% hemolysis. These higher readings appeared to be because of the darkening of the color of the oxyhemoglobin solutions. Experiments were carried out to determine to what extent this darkening took place in various glycerin and propylene glycol solutions. A solution representing 100% hemolysis was prepared by adding 1 ml. of blood to 100 ml. of distilled water. The resulting oxyhemoglobin solution was added in like amounts to each of two colorimeter tubes, one containing a measured amount of distilled water and the other containing a like amount of water–polyhydric alcohol solution. The colorimeter readings of these solutions were compared. After correcting for the absorbance of the polyhydric alcohol, per cent hemolysis readings were 2–3% higher for the water–polyhydric alcohol–blood solutions than water–blood solutions. This increase in hemolysis readings was attributed to a darkening of the red color by the polyhydric alcohols. The necessary corrections were made by subtracting this excess colorimetric reading, expressed as per cent, from the per cent hemolysis readings obtained for various glycerin and propylene glycol solutions.

Calculation of *i* Values.—When the concentrations of sodium chloride and any other compound causing the same degree of hemolysis are known, the value of *i* (isotonic coefficient) for the other compound can be calculated according to the osmotic equation used by Grosicki and Husa (2)

$$\frac{\left(\begin{matrix}i\text{ value}\\ \text{for NaCl}\end{matrix}\right)\left(\begin{matrix}\text{Gm. of NaCl}\\ \text{in 100 ml. of}\\ \text{solution}\end{matrix}\right)}{(\text{Gm.–molecular wt. of NaCl})} = \frac{\left(\begin{matrix}i\text{ value}\\ \text{for other}\\ \text{compound}\end{matrix}\right)\left(\begin{matrix}\text{Gm. of other}\\ \text{compound in 100}\\ \text{ml. of solution}\end{matrix}\right)}{\left(\begin{matrix}\text{Gm.—molecular wt. of other}\\ \text{compound}\end{matrix}\right)} \quad \text{(Eq. 1)}$$

The objective of this paper, however, was to calculate *apparent* *i* values for sodium chloride when the salt was present in a water–polyhydric alcohol system, *e.g.*, sodium chloride in 10% glycerin. In these calculations, it was necessary to assume that glycerin and propylene glycol did not contribute to the osmotic behavior of the solutions and that the sodium chloride was solely responsible for the

TABLE I.—VALUES OF *i* FOR SODIUM CHLORIDE IN VARIOUS WATER-GLYCERIN SOLUTIONS, CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF RABBIT AND HUMAN ERYTHROCYTES[a]

Glycerin, % w/v	Hemolysis, % 25	50	75	Av.
Rabbit Blood				
5	1.94	2.00	2.08	2.00
10	2.02	2.13	2.20	2.17
20	2.24	2.52	3.04	2.60
30	2.30	2.62	3.00	2.64
40	2.53	3.02	3.44	3.00
50	2.70	3.20	3.80	3.23
60	4.21	7.12	22.7	...
70	2.7	3.8	25.0	...
Human Blood				
5	1.86	1.90	1.91	1.89
10	1.88	1.92	1.95	1.92
20	1.90	1.95	1.99	1.95
30	1.96	2.02	2.07	2.02
40	2.03	2.09	2.18	2.10
50	2.08	2.16	2.25	2.16
60	2.20	2.58	3.13	2.64
70	2.28	2.87	3.81	2.99

[a] All *i* values represent an average of two blood samples.

TABLE II.—VALUES OF *i* FOR SODIUM CHLORIDE IN VARIOUS WATER-PROPYLENE GLYCOL SOLUTIONS, CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF RABBIT AND HUMAN ERYTHROCYTES[a]

Propylene Glycol, % w/v	Hemolysis, % 25	50	75	Av.
Rabbit Blood				
5	1.88	1.89	1.88	1.88
10	1.91	1.90	1.90	1.90
20	1.96	1.98	1.97	1.97
30	1.95	2.02	2.04	2.00
Human Blood				
5	1.92	1.97	2.00	1.96
10	1.91	1.94	1.95	1.93
20	1.98	1.98	1.97	1.98
30	2.05	2.09	2.05	2.06
40	1.94	1.98	1.97	1.96

[a] All *i* values represent an average of two blood samples.

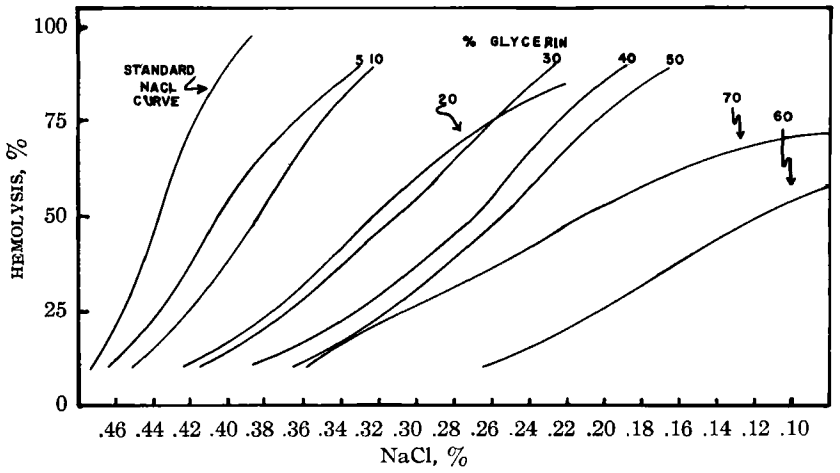


Fig. 1.—Per cent hemolysis of rabbit erythrocytes after 45 minutes at 25° in various glycerin – saline solutions.

tonicity of the solutions. Since the calculations were concerned with i values of only sodium chloride in different solvents, the molecular weights in Eq. 1 are identical and the equation becomes

$$\begin{pmatrix} i \text{ value for} \\ \text{NaCl in water} \end{pmatrix} \begin{pmatrix} \text{Gm. of NaCl} \\ \text{in 100 ml.} \\ \text{water} \end{pmatrix} = \begin{pmatrix} i \text{ value for} \\ \text{NaCl in other} \\ \text{solution} \end{pmatrix} \begin{pmatrix} \text{Gm. of NaCl} \\ \text{in 100 ml.} \\ \text{other solution} \end{pmatrix} \quad \text{(Eq. 2)}$$

The value of i for sodium chloride was taken as 1.86, which is the accepted value of i for 0.9% sodium chloride in water (2).

Curves showing the degree of hemolysis in sodium chloride–water solutions and sodium chloride–water–polyhydric alcohol solutions were plotted on rectangular coordinate paper. From these curves the concentrations of sodium chloride in Gm./100 ml. of water and the other solvent, causing 25, 50, and 75% hemolysis were determined. These values were inserted into Eq. 2, and the values of i for sodium chloride in a particular water–polyhydric alcohol solution, at concentrations giving 25, 50, and 75% hemolysis were determined. The various i values for sodium chloride in aqueous glycerin and propylene glycol solutions are shown in Tables I and II.

Preparation of Hemolysis Curves.—Fourteen experiments employing rabbit blood were carried out. The average readings of these experiments were used to construct a standard hemolysis curve (left hand side of Figs. 1 and 3). In constructing the hemolysis curves of the various polyhydric alcohol solutions, the grams of NaCl per 100 ml. of solution causing 25, 50, and 75% hemolysis were calculated with reference to the standard hemolysis curve. By utilizing Eq. 2, the Gm. of NaCl per 100 ml. in polyhydric alcohol solution causing 25% hemolysis was calculated as

$$X = \frac{A \times B}{C} \quad \text{(Eq. 3)}$$

where X = Gm. of NaCl in 100 ml. polyhydric alcohol solution causing 25% hemolysis, A = 1.86 as the i value for NaCl in water, B = Gm. of NaCl in 100 ml. of water causing 25% hemolysis (obtained from standard hemolysis curves in Figs. 1 and 3), and C = i value for NaCl in appropriate polyhydric alcohol solution (obtained from Tables I and II). Similar calculations were carried out to obtain i values at 50 and 75% hemolysis. With these three i values at 25, 50, and 75% hemolysis, the hemolysis curves for the various glycerin and propylene glycol solutions were plotted. In this manner, Figs. 1 and 3 are a compilation of the experiments run with rabbit blood, and all the curves have in common a standard hemolysis curve.

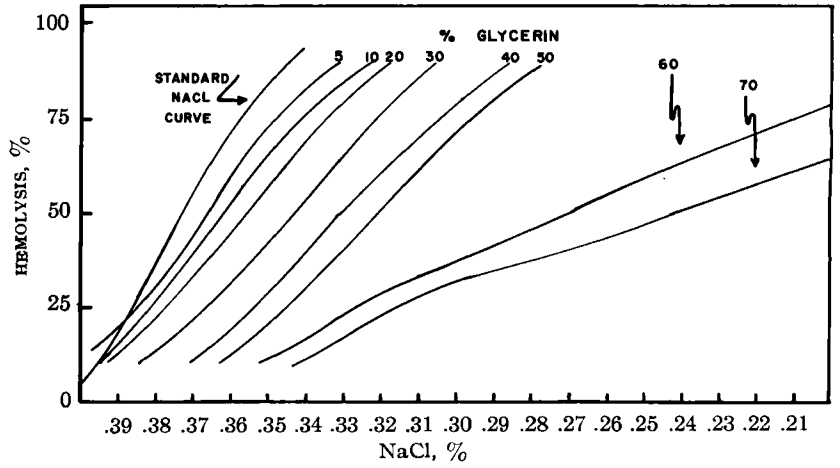


Fig. 2.—Per cent hemolysis of human erythrocytes after 45 minutes at 25° in various glycerin – saline solutions.

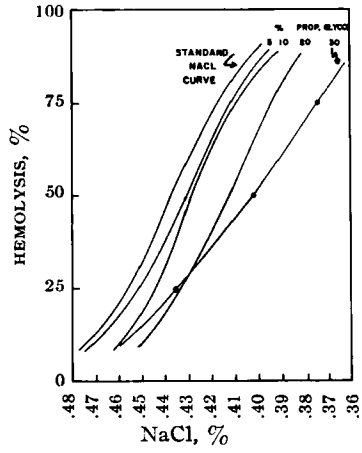


Fig. 3.—Per cent hemolysis of rabbit erythrocytes after 45 minutes at 25° in various propylene glycol–saline solutions.

Twelve similar experiments employing human blood were carried out. The average readings of these experiments were used to construct a standard hemolysis curve (left hand side of Figs. 2 and 4). The hemolysis curves for human blood in various polyhydric alcohol solutions were constructed in the manner previously described for the rabbit blood hemolysis curves. Figures 2 and 4 are a compilation of the experiments run with human blood.

RESULTS

Water-Glycerin Solutions.—The hemolysis of rabbit erythrocytes after 45 minutes at 25° in various water-glycerin solutions is shown in Fig. 5. All of the glycerin solutions void of sodium chloride caused complete hemolysis of rabbit erythrocytes except the 50, 60, and 70% solutions in which a small decrease in hemolysis was noticed.

Complete hemolysis of human erythrocytes took place in aqueous solutions containing 0.0 to 100% glycerin.

The fragility of rabbit and human erythrocytes in various water-glycerin solutions was modified or corrected by the addition of sodium chloride. It was possible to calculate *i* values for sodium chloride in various water-glycerin solutions. The average *i* values for sodium chloride in various glycerin solutions are given in Table I. In all cases, the *i* values for sodium chloride in water-glycerin solutions were greater than 1.86, the accepted value for 0.9% sodium chloride in water. This increase in *i* values can probably be attributed to some effect of glycerin on erythrocytes rather than increased activity of sodium chloride in the binary solvent. The higher *i* values for sodium chloride for rabbit blood than for human blood indicate that glycerin renders rabbit erythrocytes more resistant toward osmotic hemolysis. The sodium chloride *i* values increased with an increase in glycerin concentration which meant that less sodium chloride needed to be added to a more concentrated glycerin solution in order to make the solution isotonic to blood. The only exception to this was the higher *i* values for sodium chloride in 60% glycerin than in 70% glycerin solution when rabbit blood was employed. This can be accounted for by the fact that the least amount of hemolysis in water-glycerin solutions *sans* sodium chloride took place in 60% glycerin solution as shown in Fig. 5.

Values of *i* for sodium chloride in water-glycerin solutions containing more than 70% glycerin were not calculated because of inadequate data. Rabbit or human blood was placed in 80 and 90% glycerin solutions containing 0.2 to 0.4% sodium chloride. The opacity of these mixtures indicated that sodium chloride prevented complete hemolysis of rabbit and human erythrocytes in 80–90% glycerin solutions. The high viscosity of these solutions made it difficult to spin down the unhemolyzed cells which

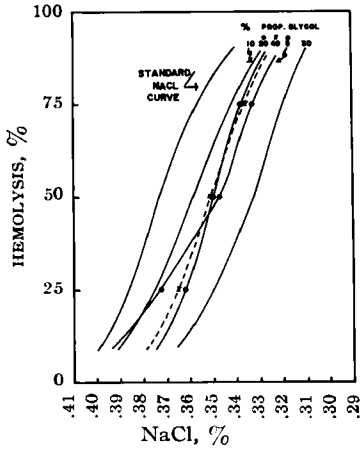
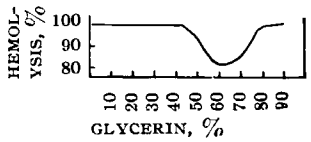


Fig. 4.—Per cent hemolysis of human erythrocytes after 45 minutes at 25° in various propylene glycol–saline solutions.

Fig. 5.—Per cent hemolysis of rabbit erythrocytes after 45 minutes at 25° in various glycerin solutions.



resulted in inaccurate colorimeter readings. After prolonged high speed centrifugation, these solutions retained a slight opaqueness.

Hemolysis curves showing the amount of laking that occurred when rabbit and human blood were added to various water–glycerin–sodium chloride solutions are shown in Figs. 1 and 2. These curves were constructed in the manner described in the *Experimental* section of this report utilizing the data presented in Table I. The addition of 0.48 to 0.28% sodium chloride to various water-glycerin solutions prevented osmotic hemolysis of rabbit erythrocytes. Hemolysis of human erythrocytes was prevented in water-glycerin solutions by the addition of 0.42 to 0.35% sodium chloride.

The average *i* values for human blood (Table I) were used to calculate the amount of sodium chloride that should be added to various water-glycerin solutions to make them isotonically equivalent to 0.9% sodium chloride solution. It was found that between 0.89 and 0.80% sodium chloride should be added to solutions containing 5–40% glycerin. The 50, 60, and 70% glycerin solutions would be made equivalent to isotonic saline solution by the addition

of 0.75, 0.63, and 0.55% sodium chloride, respectively. These data are shown in Fig. 6.

Water–Propylene Glycol Solutions.—All aqueous propylene glycol solutions ranging in concentration from 0.0 to 100% caused complete hemolysis of rabbit and human erythrocytes.

The addition of sodium chloride to water-propylene glycol solutions prevented hemolysis of rabbit erythrocytes in those solutions containing up to 30% propylene glycol. The *i* values for sodium chloride in these solutions were determined; the average values are given in Table II. The *i* values were slightly greater than 1.86. The hemolysis curves plotted from the data obtained after the addition of rabbit blood to various water–propylene glycol–sodium chloride solutions are shown in Fig. 3. Hemolysis of rabbit erythrocytes was prevented by the addition of 0.49 to 0.46% sodium chloride to 5–30% propylene glycol solutions. Peculiar hemolytic behavior of rabbit erythrocytes was noticed in 40% propylene glycol solutions. Complete protection of rabbit erythrocytes could not be achieved by the addition of sodium chloride up to concentrations of 6.0%. As the sodium chloride content increased from 0.4 to 1.0%, there was a decrease in hemolysis to a minimum of 15%; higher concentrations of sodium chloride caused an increase in hemolysis. This unusual behavior of rabbit erythrocytes in 40% propylene glycol is shown in Fig. 7. The complete hemolysis of rabbit erythrocytes in aqueous solutions containing 45% and more propylene glycol could not be prevented by the addition of up to 10.0% sodium chloride.

Hemolysis of human erythrocytes was prevented by the addition of 0.41 to 0.37% sodium chloride to 5–40% propylene glycol solutions. The *i* values for sodium chloride in these solutions were determined; the average values are given in Table II. The hemolysis curves prepared from the data obtained after the addition of human blood to various water–propylene glycol–sodium chloride solutions are illustrated in Fig. 4. Interesting behavior of human erythrocytes occurred in 45% propylene glycol solutions. There was a decrease in hemolysis in solutions containing 0.40 to 0.75% sodium chloride. Higher concentrations of sodium chloride resulted in increases in hemolysis. Complete hemolysis of human erythrocytes took place in 45% propylene glycol solutions containing 3.0% and more sodium chloride. This behavior is depicted in Fig. 8. The addition of various amounts of sodium chloride (up to 10.0%) did not prevent complete hemolysis of human erythrocytes in aqueous solutions containing 50% or more propylene glycol.

The average *i* values for human blood (Table II) were used to calculate the amount of sodium chloride that should be added to various water–propylene glycol solutions to make these solutions isotonically equivalent to 0.9% sodium chloride solution. Between 0.87 to 0.80% sodium chloride should be added to aqueous solutions containing 5–40% propylene glycol.

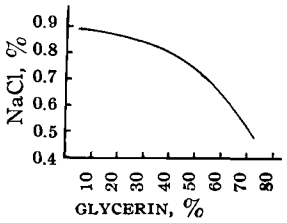


Fig. 6.—Amount of sodium chloride required to render various glycerin solutions isotonic with human blood.

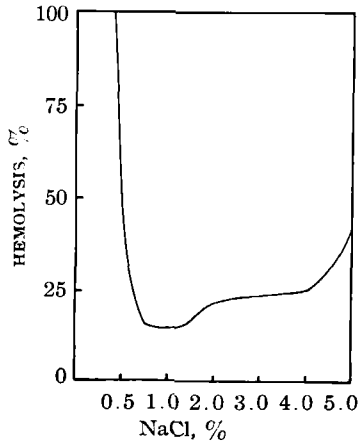


Fig. 7.—Per cent hemolysis of rabbit erythrocytes after 45 minutes at 25° in various saline solutions containing 40% propylene glycol.

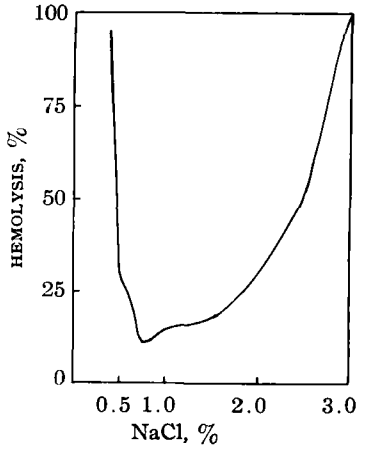


Fig. 8.—Per cent hemolysis of human erythrocytes after 45 minutes at 25° in various saline solutions containing 45% propylene glycol.

DISCUSSION

The concentrations of glycerin and propylene glycol in water that are iso-osmotic to 0.15 *M* sodium chloride and human blood, according to osmotic calculations, are 2.6 and 2.0%, respectively. The polyhydric alcohol content of aqueous solutions studied in this paper exceeded those concentrations having the same osmotic pressure as rabbit and human blood. Previous workers (1, 13) have shown that aqueous glycerin and propylene glycol solutions having concentrations that are hypo- and iso-osmotic to 0.15 *M* sodium chloride cause complete hemolysis of rabbit and human erythrocytes. Regardless of the polyhydric alcohol content, water-glycerin and water–propylene glycol solutions failed to prevent hemolysis of rabbit and human erythrocytes. These experimental data point out that when water-glycerin or water–propylene glycol are used as vehicles for intravenous solutions, the finished

product should not be assumed hypertonic with respect to blood. All aqueous glycerin and propylene glycol solutions studied in this investigation were hypotonic with respect to rabbit and human erythrocyte membranes.

Sodium chloride is effective in preventing hemolysis of rabbit and human erythrocytes in glycerin and propylene glycol solutions when the alcohols were in hypo-osmotic concentrations (1). Zanowiak and Husa (8) reported that 0.6% sodium chloride present in 10% glycerin and propylene glycol solutions prevented hemolysis of rabbit and human erythrocytes. The present investigation shows the presence of sodium chloride will prevent hemolysis of rabbit and human erythrocytes in aqueous solutions containing 5–90% glycerin. Sodium chloride prevented hemolysis of rabbit and human erythrocytes in aqueous propylene glycol solutions as long as the glycol concentration did not exceed 40–45%. Water–propylene glycol solutions containing more than 40–45% propylene glycol could not be made isotonic to rabbit and human erythrocyte membranes by the addition of sodium chloride. These data demonstrate that propylene glycol has greater hemolytic activity than glycerin. Jacobs, *et al.* (14), reported that each successive hydroxyl group added to the propane molecule decreased the rate of penetration of the alcohol into ox and rabbit erythrocytes.

Brittain and D'Arcy (15) have reported on the *in vivo* hematological effects in the rabbit following the intravenous injection of aqueous solutions containing different concentrations of propylene glycol in normal saline. Although the fragility of the red blood cells was not affected by different concentrations of propylene glycol, there was a marked decrease in blood clotting time with a corresponding increase in platelet count after the injection of 50% propylene glycol. The effect of 25% propylene glycol was considerably less. In this investigation the addition of sodium chloride to 30–40% propylene glycol solutions prevented hemolysis of rabbit and human erythrocytes, while the addition of sodium chloride to 50% propylene glycol solutions did not prevent complete hemolysis of these erythrocytes.

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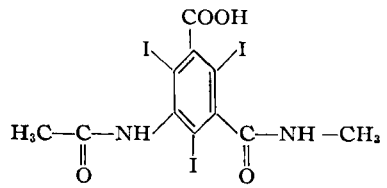
Drug Standards

Qualitative and Quantitative Tests for Iothalamic Acid

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drugs concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

5 - Acetamido - 2,4,6 - triiodo - *N* - methylisophthalamic acid, $C_{11}H_9I_3N_2O_4$; M.W. 613.92; 62.01% iodine. The structural formula of iothalamic acid may be represented as follows:



Received August 27, 1963, from the Drug Standards Laboratory, American Pharmaceutical Association Foundation, Washington, D. C. 20037.
Accepted for publication October 2, 1963.
This monograph was developed by Robert R. Stark. Mallinckrodt Chemical Works has cooperated by furnishing samples and data to aid in its development and preparation.

Physical Properties.—Iothalamic acid occurs as a white, odorless, bulky crystalline powder. In a

product should not be assumed hypertonic with respect to blood. All aqueous glycerin and propylene glycol solutions studied in this investigation were hypotonic with respect to rabbit and human erythrocyte membranes.

Sodium chloride is effective in preventing hemolysis of rabbit and human erythrocytes in glycerin and propylene glycol solutions when the alcohols were in hypo-osmotic concentrations (1). Zanowiak and Husa (8) reported that 0.6% sodium chloride present in 10% glycerin and propylene glycol solutions prevented hemolysis of rabbit and human erythrocytes. The present investigation shows the presence of sodium chloride will prevent hemolysis of rabbit and human erythrocytes in aqueous solutions containing 5–90% glycerin. Sodium chloride prevented hemolysis of rabbit and human erythrocytes in aqueous propylene glycol solutions as long as the glycol concentration did not exceed 40–45%. Water–propylene glycol solutions containing more than 40–45% propylene glycol could not be made isotonic to rabbit and human erythrocyte membranes by the addition of sodium chloride. These data demonstrate that propylene glycol has greater hemolytic activity than glycerin. Jacobs, *et al.* (14), reported that each successive hydroxyl group added to the propane molecule decreased the rate of penetration of the alcohol into ox and rabbit erythrocytes.

Brittain and D'Arcy (15) have reported on the *in vivo* hematological effects in the rabbit following the intravenous injection of aqueous solutions containing different concentrations of propylene glycol in normal saline. Although the fragility of the red blood cells was not affected by different concentrations of propylene glycol, there was a marked decrease in blood clotting time with a corresponding increase in platelet count after the injection of 50% propylene glycol. The effect of 25% propylene glycol was considerably less. In this investigation the addition of sodium chloride to 30–40% propylene glycol solutions prevented hemolysis of rabbit and human erythrocytes, while the addition of sodium chloride to 50% propylene glycol solutions did not prevent complete hemolysis of these erythrocytes.

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Drug Standards

Qualitative and Quantitative Tests for Iothalamic Acid

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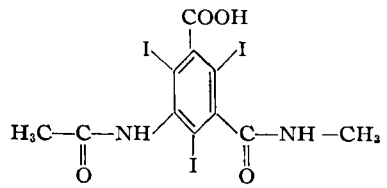
5 - Acetamido - 2,4,6 - triiodo - *N* - methylisophthalamic acid, $C_{11}H_9I_3N_2O_4$; M.W. 613.92; 62.01% iodine. The structural formula of iothalamic acid may be represented as follows:



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Accepted for publication October 2, 1963.
This monograph was developed by Robert R. Stark. Mallinckrodt Chemical Works has cooperated by furnishing samples and data to aid in its development and preparation.

Physical Properties.—Iothalamic acid occurs as a white, odorless, bulky crystalline powder. In a

melting point capillary it darkens at about 275° and decomposes with evolution of iodine fumes at 281–285° (U.S.P. XVI Class Ia). It is slightly soluble in water and in alcohol, and is practically insoluble in chloroform. It is soluble in solutions of alkali hydroxides.

Identity Tests.—Heat about 500 mg. of iothalamic acid in a porcelain crucible over a free flame: violet vapors of iodine are evolved.

A 1:100,000 solution of iothalamic acid in alcohol exhibits an ultraviolet absorbance maximum at about 242 mμ [absorptivity (1%, 1 cm.) about 530]. The spectrum is shown in Fig. 1.

The infrared spectrum of a 0.5% dispersion of iothalamic acid in potassium bromide, in a disk of about 0.82-mm. thickness is shown in Fig. 2.

Purity Tests.—Dry about 1 Gm. of iothalamic acid, accurately weighed, at 105° for 18 hours: it loses not more than 1.0% of its weight.

Char about 1 Gm. of iothalamic acid, accurately weighed, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.1%.

Add about 200 mg. of iothalamic acid to a mixture of 2 ml. of water and 2 ml. of chloroform, shake vigorously, and allow the layers to separate: the chloroform layer remains colorless (absence of free iodine).

Dissolve 500 mg. of iothalamic acid in 20 ml. of water containing 2–3 drops of stronger ammonia

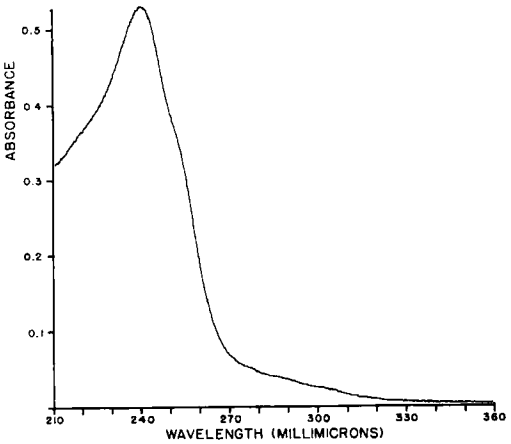

Fig. 1.—Ultraviolet absorption spectrum of iothalamic acid in alcohol (10 mcg. per ml.); Beckman model DK-2A spectrophotometer.

water contained in a 150-ml. glass-stoppered flask. Add 5 ml. of diluted nitric acid, shake vigorously, and filter the mixture through chloride-free paper. Add 2 ml. of silver nitrate T.S. to the filtrate: the solution shows no more turbidity than that produced by 0.01 mg. of chloride ion (Cl) in an equal volume of solution containing 5 ml. of diluted nitric acid and 2 ml. of silver nitrate T.S. (about 20 parts per million).

Transfer 1 Gm. of iothalamic acid to a 250-ml. beaker, add 15 ml. of nitric acid, and evaporate almost to dryness on a hot plate. Cool, add 10 ml. of nitric acid and 5 ml. of perchloric acid, and evaporate to dryness. Cool, add 2 ml. of hydrochloric acid, and rinse down the inner walls of the beaker with a few milliliters of water. Evaporate to dryness, cool, add another 2 ml. of hydrochloric acid, and again rinse down the inner walls of the beaker with a few milliliters of water. Evaporate to dryness, and bake the residue for a few minutes. Cool, add 10 ml. of water, 1 drop of phenolphthalein T.S., and sodium hydroxide T.S., dropwise, until the solution just turns pink. Add 1*N* hydrochloric acid dropwise until the color is discharged, add 1 ml. of diluted acetic acid, and dilute to 25 ml. with water. Prepare a control containing 20 mcg. of lead (Pb) and 1 ml. of diluted acetic acid in 25 ml. Add a small amount of activated charcoal (Darco G-60 is satisfactory) to each solution, and filter through retentive paper into 50-ml. color comparison tubes. Wash with a few ml. of water, dilute the solutions to 40 ml., and add 10 ml. of hydrogen sulfide T.S. to each solution: any brown color produced in the sample solution is not darker than that in the control solution: the heavy metals limit for iothalamic acid is 20 parts per million.

Assay.—(*Iodine*).—Transfer about 300 mg. of iothalamic acid, previously dried at 105° for 18 hours and accurately weighed, to a glass-stoppered 250-ml. flask, add 30 ml. of sodium hydroxide solution (1 in 20) and 500 mg. of powdered zinc. Connect the flask to a reflux condenser and reflux the mixture for 30 minutes. Cool to room temperature, and wash the condenser with 20 ml. of water. Filter the mixture and wash the flask and the filter with small portions of water, adding the washings to the filtrate. Add to the filtrate 5 ml. of glacial acetic acid and 1 ml. of a 1 in 1000 solution of tetrabromophenolphthalein ethyl ester in glacial acetic acid, and titrate with 0.05 *N* silver nitrate until the color of the yellow precipitate just changes to green. Each milliliter of 0.05*N* silver

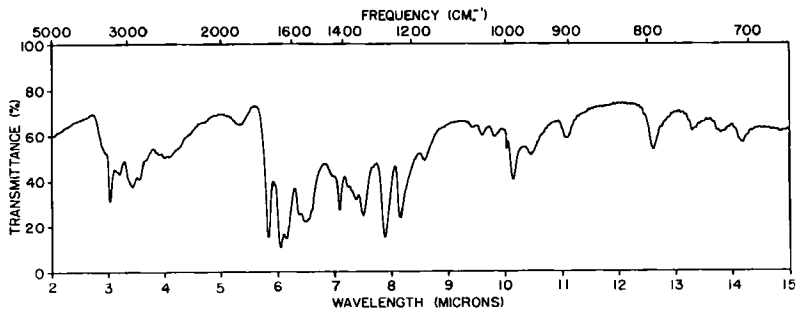

Fig. 2.—Infrared spectrum of iothalamic acid in potassium bromide disk (0.5%); Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

nitrate is equivalent to 6.345 mg. of iodine (I). The amount of iodine found is not less than 61.2% and not more than 62.8% of the weight of the sample taken.

(*Iothalamic Acid*).—Transfer about 2 Gm. of iothalamic acid, previously dried at 105° for 18 hours and accurately weighed, to a 250-ml. conical flask, and add 50.0 ml. of 0.1*N* sodium hydroxide. Swirl the flask to dissolve the sample, add 3 drops of phenolphthalein T.S., and titrate the excess base with 0.1 *N* hydrochloric acid. Each ml. of 0.1 *N* sodium hydroxide consumed is equivalent to 61.39 mg. of $C_{11}H_9I_3N_2O_4$. The amount of iothalamic acid found is not less than 99.0% and not more than 101.0% of the weight of the sample taken.

DOSAGE FORMS OF IOTHALAMATE SALTS

Meglumine Iothalamate Injection

A sterile solution of iothalamic acid in water for injection, prepared with the aid of meglumine (*N*-methylglucamine); $C_{18}H_{26}I_3N_3O_9$; M.W. 809.14.

Physical Properties.—Meglumine iothalamate injection is a clear, pale yellow, slightly viscous liquid. The pH is between 6.8 and 7.5.

Identity Tests.—Dilute a volume of meglumine iothalamate injection, equivalent to about 2 Gm. of meglumine iothalamate, with water to 100 ml., add 5 ml. of dilute hydrochloric acid, mix, and filter off the precipitated iothalamic acid. Wash the precipitate with four 10-ml. portions of water, and dry at 105° for 4 hours: the dried residue responds to the chemical and infrared identity tests for iothalamic acid.

Purity Tests.—Transfer a volume of meglumine iothalamate injection, equivalent to 1 Gm. of meglumine iothalamate, to a 250-ml. beaker. Proceed as directed in the heavy metals test in the monograph for iothalamic acid, beginning with ". . .add 15 ml. of nitric acid. . . ." The heavy metals limit for meglumine iothalamate is 20 parts per million.

To about 2 ml. of meglumine iothalamate injection add a few drops of starch T.S.: no blue color is produced (absence of free iodine).

Assay.—(*Meglumine Iothalamate*).—Pipet a volume of meglumine iothalamate injection, equivalent to about 3 Gm. of meglumine iothalamate, into a 100-ml. volumetric flask, add water to volume, and mix. Pipet 10 ml. of the solution into a 250-ml. glass-stoppered flask and proceed as directed in the *Assay for Iodine* under iothalamic acid, beginning with ". . .add 30 ml. of sodium hydroxide solution (1 in 20). . . ." Each milliliter of 0.05 *N* silver nitrate is equivalent to 13.49 mg. of $C_{18}H_{26}I_3N_3O_9$. The amount of meglumine iothalamate found is not less than 96.0% and not more than 104.0% of the labeled amount.

Sodium Iothalamate Injection

A sterile solution of iothalamic acid in water for injection, prepared with the aid of sodium hydroxide.

Physical Properties.—Sodium iothalamate injection is a clear, pale yellow, slightly viscous liquid. The pH is between 6.8 and 7.5; $C_{11}H_8I_3N_2NaO_4$; M. W. 635.90.

Identity Tests.—The solution responds to the identity tests for meglumine iothalamate injection and to the U.S.P. XVI flame test for sodium.

Purity Tests.—Determine the heavy metals content by the procedure for meglumine iothalamate injection. The heavy metals limit for sodium iothalamate is 20 parts per million.

To about 2 ml. of sodium iothalamate injection add a few drops of starch T.S.: no blue color is produced (absence of free iodine).

Assay.—(*Sodium Iothalamate*).—Assay sodium iothalamate injection by the procedure for meglumine iothalamate injection. Each milliliter of 0.05 *N* silver nitrate is equivalent to 10.60 mg. of $C_{11}H_8I_3N_2NaO_4$. The amount of sodium iothalamate found is not less than 97.5% and not more than 102.5% of the labeled amount.

DISCUSSION

U.S.P. and N.F. terminology for solubility, melting range, reagents, etc., have been used wherever feasible.

Iothalamic acid, synthesized by Hoey, *et al.* (1), is a radiographic contrast medium. Solutions of its salts are used for intravenous urography (meglumine iothalamate)[1] and for intravascular angiocardiography and aortography (sodium iothalamate).[2] Chemically, these solutions are similar to methylglucamine diatrizoate injection U.S.P. XVI, and sodium diatrizoate injection U.S.P. XVI, respectively, and the tests and standards in the above monographs are also similar to those in the official monographs.

Identity Tests.—Because of similarity of structure, identity tests based upon evolution of iodine vapor and comparison of the ultraviolet absorption spectrum with that of a reference standard are not sufficient to identify iothalamic acid or its salts. Comparison of the infrared spectrum with that produced by reference material under identical conditions provides a satisfactory test.

Quantitative Methods.—The quantitative methods provided are simple enough to perform and possess sufficient accuracy and precision for their intended purposes. Acidimetric determination of iothalamic acid gave an average value of 100.3 ± 0.2%.[3] Argentimetric determination of this compound gave an average value equivalent to 61.1 ± 0.1% iodine. Analysis of commercial preparations gave average values of 98.3 ± 0.1% and 97.7 ± 0.1% for meglumine iothalamate and sodium iothalamate injections, respectively.

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[1] The tradename for meglumine iothalamate injection is Conray.

[2] The tradename for sodium iothalamate injection is Angio-Conray.

[3] Maximum deviation from the mean value.

Dosage Variation in Compressed Tablets

By C. D. SMITH, T. P. MICHAELS, M. J. CHERTKOFF, and L. P. SINOTTE

A study was undertaken to evaluate the intertablet dosage variation in a number of different products by the use of automated instrumentation. Representative samples of actual production runs were used; an analysis of the data is presented.

ALTHOUGH PRODUCT DOSAGE is expressed on an individual compressed tablet basis, potency testing utilizes an analytical sample which is a composite of many tablets. An assay procedure will often read: "Weigh and finely powder not less than 20 ——— tablets. Weigh accurately a portion of the powder, equivalent to about . . ." (1). Such procedures give information on the average tablet assay; however, they give no information on the individual tablets. For example, if half of the tablets were 50% high in potency and half 50% low, the composite sample would still assay satisfactorily.

Considerable interest has recently been shown in studying this topic as exemplified in reports by Moskalyk, *et al.* (2), Garrett (3), and Evers (4). Suggestions have been made to modify the presently accepted practice of assaying a composite sample and to assay individual tablets (5, 6). Because of the difficulty of performing large numbers of assays, these studies have been limited to a relatively small sample, usually less than 100, which may not be representative of modern large scale production lots. For this reason, the nature of the distribution of individual tablet assays has not been established. Such information is necessary before the need for a change in existing procedures can be established and corrective action devised.

Recent work in this laboratory has made available automated chemical instrumentation (7–9) capable of automatically analyzing 20 tablets per hour. This facilitates the collection of a large number of assays. This paper presents assay and weight variation data on round, discoid, individual tablets—selected to be representative of different production batches of several products—in an attempt to define the nature of the distribution of dosage variation in commercially available compressed tablets.

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TABLE I.—VARIATION RANGE OF INDIVIDUAL TABLET ASSAYS

Product	Lot	Tablets Assayed, No.	% Tablets Outside 90–110%	% Tablets Outside 85–115%
7	N	314	0.64	0.00
	O	282	0.36	0.00
4	D	277	0.72	0.00
	E	311	0.32	0.00
6	C	252	0.00	0.00
	D	375	0.27	0.00
3	C	267	0.00	0.00
	D	273	0.73	0.00
8	A	264	0.00	0.00
	B	303	0.66	0.00
		2918	Av. 0.37	0.00

EXPERIMENTAL

Chemical assays were performed with modified AutoAnalyzer[1] as described by Michaels and Sinotte (7) and Holl and Walton (8). Individual tablets, taken at approximately uniform intervals throughout the compression of a lot, were assayed automatically. The assay procedures employed were colorimetric as described by Wrightman and Holl (9). The individual tablet weights were obtained by standard analytical balances or the Mettler automatic tablet weigher.[2] All curves in the figures are fitted by eye.

RESULTS AND DISCUSSION

Variation Range of Individual Tablet Assays.—Table I summarizes the assay results obtained on two batches of each of five different products. These data are typical of other products and batches studied over a 1-year period. These results were tested for conformance with the presently accepted composite limits of ±10% (1) and against somewhat wider limits of ±15%, as recently suggested (5). Over 99% of the tablets conform to presently accepted composite limits; only a few minor deviations were found. All tablets conform to the wider limits.

Distribution of Individual Tablet Assay.—To define the distribution of individual tablet assays

[1] T. M. Technicon Controls, Inc., Chauncey, N. Y.
[2] Mettler Instrument Corp., Princeton, N. J.

TABLE II.—DISTRIBUTION OF INDIVIDUAL TABLET ASSAYS

Product	Lot	Active Ingredient, %	Tablets Assayed, No.	$\bar{X}$ in % Label Claim	σ in % Label Claim	α_3[a]	α_4[a]	Curve, Type
1	A	23.8	116	101	2.8	−0.12	3.86	Normal
	B		58	103	3.8	0.05	4.14	Normal
	C		74	98	2.6	−0.42	4.04	Normal
2	A	23.3	88	101	3.4	0.13	3.05	Normal
	B		90	101	2.9	0.09	2.86	Normal
3	A	23.3	104	102	2.7	0.19	2.53	Normal
	B		95	102	2.4	−0.06	3.42	Normal
4	A	23.3	93	102	3.9	0.36	3.16	Normal
	B		108	102	3.4	0.21	2.30	Normal
	C		100	102	3.8	[−0.58]	2.74	Skewed—low side
5	A	68.5	102	98	2.3	[−0.73]	3.34	Skewed—low side
	B		100	100	2.0	0.10	3.75	Normal
	C		95	102	2.8	−0.17	3.81	Normal
6	A	88.5	101	101	2.8	0.29	[4.40]	Leptokurtic
	B		91	100	1.7	−0.46	4.01	Normal
7	A	90.7	105	101	2.1	0.47	3.30	Normal
	B		181	99	1.4	[−0.39]	2.94	Skewed—low side
	C		49	100	4.5	[−1.00]	3.42	Skewed—low side
	D		100	99	1.7	−0.14	3.02	Normal

[a] Tested for significance by student "t" test at 0.05 level; significant values indicated by brackets.

further lots were studied. The curves are described numerically by $\bar{X}$ (average assay), σ (standard deviation), α_3 (a measure of skewness), and α_4 (a measure of kurtosis).

The expected value for α_3 is zero for a normal distribution. A negative value indicates distribution skewed to the left; a positive value indicates a distribution skewed to the right. The expected value for α_4 is 3 for a normal distribution. A value greater than 3 indicates a leptokurtic distribution (more peaked than normal); a value less than 3 indicates a platykurtic distribution (flatter than normal)(10).

It is obvious from the data in Table II that the average assays are very close to label claims. The standard deviations are small which confirms the information reported in Table I and, with few exceptions, the curves are essentially normal. The significance of the α_3 and α_4 values were determined (11), and the type of curve is indicated.

Examination of the data indicates a relationship between standard deviation and per cent of active ingredient in the tablet. Figure 1 shows that as the per cent of active ingredient increases, the variation in individual assays decreases.

The standard deviation of 4.5 (Product 7, Lot C) is an aberrant value and may be due to experimental errors. To confirm that this is not a normal situation, nine more lots of Product 7 were investigated (see Table III). The average standard deviation of these nine lots is 1.7, which lies very close to the estimated line.

Distribution of Individual Tablet Weights.—To define the distribution of individual tablet weights 13 lots representing six different products were studied. The data in Table IV are typical of many other products and lots studied.

Examination indicates that the data are essentially normal as discussed above. The average weights are close to the target weight and the standard deviations are small. These standard deviations in milligrams vary directly with tablet weight as shown in Fig. 2. However, when the relative per cent error ($\sigma/\bar{X} \times 100$) is plotted against average weight (Fig. 3) it is evident that the relative per cent error remains constant at about 1.4 for tablets weighing more than 300 mg. Further work is indicated to study the rise in relative per cent error for tablets weighing less than 300 mg.

Correlation Between Individual Tablet Weight and Assay.—To study the relationship between the two variables, individual tablets from 12 lots representing six different products were weighed and then assayed. The distribution of these two variables have been previously shown in Tables II and IV. The relationship of these two variables is expressed as correlation coefficients. There is no relationship when the value is zero, whereas perfect

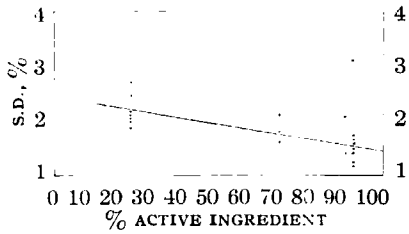

Fig. 1.—Standard deviation (assays) *vs.* per cent active ingredient.

TABLE III.—INDIVIDUAL TABLET ASSAY STANDARD DEVIATION ON ADDITIONAL LOTS OF PRODUCT 7

Lot	Tablets Assayed, No.	$\bar{X}$ in % Label Claim	σ in % Label Claim	Relative Error $\sigma/\bar{X} \times 100$
E	234	100.3	2.2	2.2
F	180	100.5	1.8	1.8
G	91	100.7	2.0	2.0
H	51	101.1	2.0	2.0
I	75	99.3	2.1	2.1
J	79	99.5	1.7	1.7
K	75	99.6	1.3	1.3
L	55	100	1.3	1.3
M	88	100	2.0	2.0
			Av. 1.7	1.7

TABLE IV.—DISTRIBUTION OF INDIVIDUAL TABLET WEIGHTS

Product	Lot	Tablets Weighed, No.	Target Wt.	$\bar{X}$ mg.	σ mg.	$\sigma/\bar{X} \times 100$	α_3[a]	α_4[a]	Curve, Type
1	A	116	105.0	105	2.1	2.0	−0.14	2.51	Normal
	C	74		105	3.3	3.1	−0.06	[4.93]	Leptokurtic
2	A	88	107.5	101	3.4	3.4	0.41	[6.31]	Leptokurtic
	B	90		107	2.2	2.1	0.12	[5.16]	Leptokurtic
3	A	104	215.0	213	2.9	1.4	0.07	3.11	Normal
	B	95		215	3.8	1.8	−0.48	[4.07]	Leptokurtic
5	A	102	365.0	361	6.1	1.7	[−1.24]	[5.84]	Leptokurtic and skewed —low side
	B	100		362	4.5	1.2	[−0.67]	[4.36]	Leptokurtic and skewed —low side
	C	95		372	5.9	1.6	0.02	3.69	Normal
6	A	101	565.0	563	8.6	1.5	−0.10	2.29	Normal
	B	91		564	7.1	1.3	0.09	2.80	Normal
7	A	105	551.0	560	5.8	1.0	0.10	2.54	Normal
	D	100		552	6.4	1.2	0.29	2.12	Normal
9	A	100	713.0	718	9.1	1.3	0.05	2.32	Normal
	B	100		706	7.2	1.0	0.16	2.48	Normal
	C	100		706	7.7	1.1	0.14	2.93	Normal
	D	300		713	10.2	1.4	0.09	[2.31]	Platykurtic
10	A	1001	648.0	656	9.6	1.5	0.15	2.83	Normal
	B	255		646	5.9	0.9	0.03	2.89	Normal
	C	273		648	7.0	1.1	[0.40]	[3.44]	Leptokurtic and skewed —high side
	D	293		648	9.6	1.5	0.24	2.73	Normal

[a] Tested for significance by student "t" test at 0.05 level; significant values indicated by brackets.

positive correlation exists when the value is one. The results in Table V show a significant correlation in 10 out of 12 lots at a low level, in the range studied. In the range studied there seems to be no relationship between weight-assay correlations and per cent of active ingredient in the tablet.

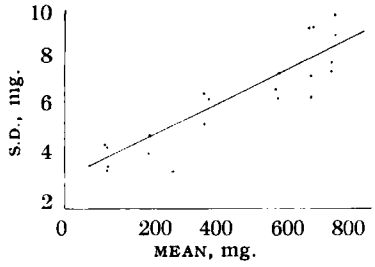

Fig. 2.—Standard deviation (weights) *vs.* mean.

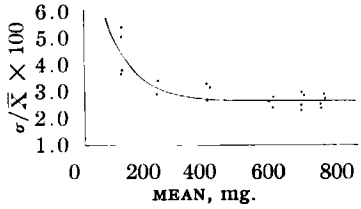

Fig. 3.—Relative error (weights) *vs.* mean.

CONCLUSIONS

It was found from the sample studied in our laboratories that (*a*) the spread of dosage variation in individual compressed tablets was very small, (*b*) the distribution of individual tablet weights and assays was essentially normal; and (*c*) a correlation existed between tablet weight and assay, at a low level, in the range studied.

In this type of situation where extensive research.

TABLE V.—CORRELATION BETWEEN INDIVIDUAL TABLET WEIGHT AND ASSAY

Product	Lot	Tablets, No. (Wt. and Assay)	Correlation Coefficient	Correlation Significance	% Active Ingredient by Wt.
1	A	116	−0.16	$p > 0.05$ N.S.	23.8
	C	74	0.35	$p < 0.01$ S	23.8
2	A	88	0.46	$p < 0.001$ S	23.3
	B	90	0.22	$p < 0.05$ S	23.3
3	A	104	0.40	$p < 0.001$ S	23.3
	B	95	0.24	$p < 0.05$ S	23.3
5	A	102	0.55	$p < 0.001$ S	68.5
	B	100	0.18	$p > 0.05$ N.S.	68.5
	C	95	0.44	$p < 0.001$ S	68.5
7	A	105	0.27	$p < 0.01$ S	90.7
	D	100	0.47	$p < 0.001$ S	90.7
6	B	91	0.34	$p < 0.001$ S	88.5

considerable production experience, and sufficient in-process controls exist, low dosage variation in individual compressed tablets may be achieved. Modern quality control concepts emphasize that quality should be built into the product rather than tested in. The results of this study indicate what can be achieved by following this concept.

It should be noted that the tablets studied were round, discoid shape, containing more than 20% active ingredient, and the assay procedures involved have a high degree of reliability. Further studies should be performed with tablets of irregular shapes and lower percentage of active ingredient. It would also be interesting to examine the effects of assay procedures of greater variability on this type of study.

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Notes

Synthesis of Selected Amides of Mono- and Bis(carboxypiperidino)alkanes

By RONALD P. QUINTANA and WAYNE A. SHRADER

The synthesis of selected amides of mono- and bis(carboxypiperidino)alkanes is reported.

THE SYNTHESIS of selected amides of mono- and bis(carboxypiperidino)alkanes has been undertaken to expand significantly those series of compounds previously reported (1–4). The compounds described in this communication were chosen on the basis of enzymodynamic studies, wherein particularly interesting variations in biochemical response relative to modifications in chemical constitution were noted and parallel effects upon surface and interfacial tension were observed. The fact that in some instances, among the monomethyl-, dimethyl-, monoethyl-, and diethylcarboxamido derivatives, the latter was the only one effecting appreciable or even significant inhibition in isolated human plasma "pseudo"-cholinesterase systems (5), prompted us to explore variations in the amide group in terms of steric factors and the electrophilic character of the carbonyl carbon. Since the mono- and bis[3-(N,N-diethylcarboxamido)piperidino]-ethanes and decanes reflected perhaps the most interesting relationships between molecular constitution, cholinesterase inhibition (5), and surface and interfacial tension (6), these four analogs were selected as model molecules for this study.

In general, the synthetic procedures employed in this investigation were those utilized by Lassio and co-workers (1, 2, 4). The compounds 1-ethyl-3-(N,N-diethylcarboxamido)-1,2,5,6-tetrahydropyridine hydrochloride (XVIII) and 1-decyl-3-(N,N-diethylcarboxamido)-1,2,5,6-tetrahydropyridine hydrochloride (XIX) were synthesized by sodium borohydride reduction of the appropriate pyridinium salts, a method employed by Lyle and co-workers (7) in the preparation of arecoline (methyl 1-methyl-1,2,5,6-tetrahydronicotinate) and methyl 1-methyl-1,2 5,6-tetrahydroisonicotinate.

The position of the double bond in compounds XVIII and XIX was confirmed by a comparison of the ultraviolet and infrared spectra of these compounds with the corresponding spectra of 1-methyl-3-(N,N-diethylcarboxamido)-1,2,5,6-tetrahydropyridine hydrochloride (XX) (1), prepared from arecoline (see Table I). Lyle's recent interpretation (8) of the mechanism involved in the sodium borohydride reduction of pyridinium ions provides further substantiation in this regard.

EXPERIMENTAL

4-(N,N-Diethylcarboxamido)pyridine (I).—This

TABLE I.—ULTRAVIOLET AND INFRARED SPECTRA

Compd. No.	Infrared Spectra, μ[a]	Ultraviolet Spectra[b] $\lambda_{max.}$ mμ	ϵ
XVIII	5.99 (m),[c] 6.20 (s)[d,e]	206	7800
XIX	5.99 (m),[c] 6.20 (s)[d,e]	206	8100
XX[f]	5.99 (m),[c] 6.20 (s)[d,e]	206	7880

[a] Infrared spectra were run in chloroform. [b] Ultraviolet spectra were run in ethanol by Huffman Microanalytical Laboratories, Wheatridge, Colo. [c] Attributed to the double bond. [d] Attributed to the conjugated amide carbonyl function. [e] Band is broad. [f] Lasslo, *et al.* (1).

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EXPERIMENTAL

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TABLE I.—ULTRAVIOLET AND INFRARED SPECTRA

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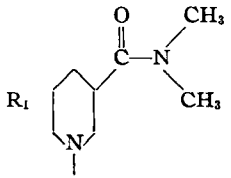
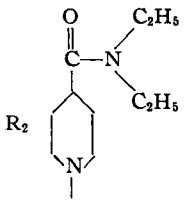
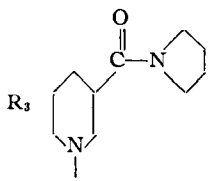
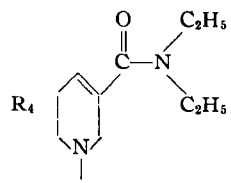
TABLE II.—COMPOUNDS PREPARED BY PROCEDURES DESCRIBED









Compd. No.	Alkane	Substitution	Prepn., Method	Yield, %[b]	Salt	Recrystn. Solvent[c]	M.p., °C.[d]	Analyses[a] C, % Calcd.	C, % Found	H, % Calcd.	H, % Found	Br, % Calcd.	Br, % Found	Cl, % Calcd.	Cl, % Found	N, % Calcd.	N, % Found
III	Ethane	1-(R_1)	A	71.6	HBr	E-EA	218.0–218.5	45.29	45.47	7.98	7.81	30.13	30.13	...	...	10.56	10.58
V	Decane	1-(R_1)	A	66.4	HBr	E-EA	167.0–167.3	57.28	57.20	9.88	10.09	21.17	21.00	...	...	7.42	7.56
VI	Ethane	1,2-Bis(R_1)	B	50.2	2HBr	M-EA	268.6–268.9[e]	43.21	43.33	7.25	7.33	31.94	32.06	...	...	11.20	11.40
VII	Decane	1,10-Bis(R_1)	B	73.7	2HBr	E-EA	196.0–199.0	50.98	50.78	8.56	8.49	26.09	26.22	...	...	9.15	8.90
VIII	Ethane	1-(R_2)	A	60.8	HBr[f]	E-EA	220.8–221.0	49.15	49.15	8.59	8.31	27.25	27.20	...	...	9.55	9.51
IX	Decane	1-(R_2)	A	68.7	HBr	E-EA	200.0–200.8[e]	59.24	59.39	10.19	10.12	19.71	19.65	...	...	6.91	6.86
X	Ethane	1,2-Bis(R_2)	B	48.9	2HBr	M	300.0–300.5[e]	47.49	47.44	7.97	8.02	28.72	28.84	...	...	10.07	9.88
XI	Decane	1,10-Bis(R_2)	B	65.8	2HBr	E-EA	253.0–253.2[e]	53.89	53.68	9.05	8.80	23.90	23.77	...	...	8.38	8.33
XII	Ethane	1-(R_3)	A	74.2	HBr	E-EA	163.0–163.7	49.49	49.38	7.96	8.09	27.44	27.20	...	...	9.62	9.51
XIII	Decane	1-(R_3)	A	87.4	HBr	E-EA	161.8–162.4	59.54	59.70	9.74	9.49	19.81	19.55	...	...	6.94	6.90
XIV	Ethane	1,2-Bis(R_3)	B	15.0	2HBr	E-EA	268.7–269.3[e]	47.83	47.60	7.30	7.50	28.93	28.74	...	...	10.14	10.42
XV	Decane	1,10-Bis(R_3)	B	39.2	2HBr	E-EA	232.0–234.0[e]	54.21	54.19	8.49	8.66	24.05	24.15	...	...	8.43	8.62
XVIII	Ethane[g]	1-(R_4)	C	29.5	HCl[h]	E-EA	116.5–117.0	58.40	58.51	9.39	9.45	...	...	14.37	14.34	11.35	11.20
XIX	Decane[g]	1-(R_4)	C	15.8	HCl	EA-EE	132.7–133.3	66.91	66.72	10.95	11.02	...	...	9.88	10.06	7.80	7.85

[a] Analyses by Drs. G. Weiler and F. B. Strauss, Oxford, England. [b] Crude yield. [c] E, Ethanol; EA, ethyl acetate; EE, ethyl ether; M, methanol. [d] Melting points uncorrected. [e] Decomposition. [f] Lit. (11) m.p. for HI 124–126°. [g] The synthesis of the corresponding bis-substituted derivative is in progress. [h] Free base, b.p. 102–104°/0.27 mm. Hg.

compound was prepared from isonicotinic acid. The product distilled at 94°/0.15 mm. Hg, uncorrected (lit. (9) b.p. 123 to 123.5°/3 mm. Hg, corrected; lit. (10) b.p. 109°/0.5 mm. Hg, uncorrected).

3-(Pyrrolidinoformyl)pyridine (II).—This compound was prepared from nicotinic acid. The product distilled at 114°/0.04 mm. Hg, uncorrected (lit. (10) b.p. 131–133°/0.3 mm. Hg, uncorrected).

The compounds listed in Table II were prepared by the following procedures.

Procedure A: **1-Ethyl-3-(N,N-dimethylcarboxamido)piperidine Hydrobromide** (III).—N,N-Dimethylnicotinamide (25 Gm., 0.167 mole) (IV) and ethyl bromide (48.3 Gm., 0.443 mole) were dissolved in 200–300 ml. anhydrous benzene and refluxed for 48–60 hours. The precipitate formed in the reaction was separated and was dissolved in 150–200 ml. distilled water. The aqueous solution was washed with benzene, treated with charcoal, and filtered. The filtrate was subjected to hydrogenation (aqueous or hydroalcoholic solution) in the presence of 1.0 Gm. platinum oxide (Adams' catalyst) at maximum pressures of 45–50 p.s.i. After absorption of hydrogen ceased, the platinum oxide was filtered off and the solvent was removed by azeotropic distillation under reduced pressure with absolute ethanol and anhydrous benzene. The product was purified by recrystallization.

Procedure B: **1,2-Bis[3-(N,N-dimethylcarboxamido)piperidino]ethane Dihydrobromide** (VI).—N,N-Dimethylnicotinamide (52.1 Gm., 0.347 mole) (IV) and 1,2-dibromoethane (32.6 Gm., 0.174 mole) were dissolved in 200 ml. anhydrous benzene and refluxed for 53–62 hours. The precipitate formed during the reaction was then treated as described under *Procedure A*.

Procedure C: **1-Ethyl-3-(N,N-diethylcarboxamido)-1,2,5,6-tetrahydropyridine Hydrochloride** (XVIII).—1 - Ethyl - 3 - (N,N - diethylcarboxamido)pyridinium bromide (XVII) (2) was prepared by refluxing N,N-diethylnicotinamide (40 Gm., 0.224 mole) (XVI) and ethyl bromide (61 Gm., 0.560 mole) in 250 ml. anhydrous benzene for 48 hours. Excess alkyl halide and solvent were removed by decantation. The residual oil was dissolved in 150 ml. water, the aqueous solution was washed with benzene, and the water was removed under reduced pressure. Last traces of moisture were removed by azeotropic distillation with benzene. The oily product (60 Gm., 0.209 mole) was dissolved in 400 ml. dry methanol and cooled in an ice bath. Sodium borohydride (31.7 Gm., 0.838 mole) was added with stirring to the cold solution over 30 minutes; subsequently the solution was stirred an additional 30 minutes. Methanol was removed by distillation under reduced pressure to give a spongy yellow residue which was dissolved in 230 ml. water and saturated with potassium carbonate. The mixture was extracted with ether, the combined ether extracts were dried over anhydrous potassium carbonate, filtered, and the ether was removed by distillation. The oily residue was purified by conversion to the hydrochloride (anhydrous HCl in anhydrous ether) with or without prior fractionation under reduced pressure. Residual traces of moisture were removed from the oily hydrochloride by azeotropic distillation with benzene. The salt was then purified by recrystallization from ethanol-ethyl acetate.

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Sensitive and Reproducible Assay Method for Chymotrypsin

By B. L. KABACOFF, M. UMHEY, A. WOHLMAN, and S. AVAKIAN

A new assay method for chymotrypsin was developed. This method, based on N-acetyl-L-tyrosine ethyl ester (ATEE), was found to be ten times as sensitive as the proposed N.F. assay method for chymotrypsin. In the new method described, the residual substrate is measured by a single colorimetric determination. The method is convenient and highly reproducible.

THE CLASSICAL assay methods for proteolytic activity such as hemoglobin digestion, casein digestion, and milk clotting, although convenient and simple, lack specificity. N-acetyl-L-tyrosine ethyl ester (ATEE) is a specific substrate for chymotrypsin. The proposed N.F. method for the analysis of chymotrypsin, based on the work of Schwert and Takenaka (1), utilizes ATEE as the enzyme substrate. The rate of hydrolysis of this substrate in pH 7.0 buffer is followed spectrophotometrically at 237 mμ. Readings are taken every 30 seconds for a period of 4 minutes. The temperature must be kept within ±0.1° of 25.0°.

The above method has certain drawbacks which limit its practical application. Elaborate equipment is required to maintain the cell compartment of the spectrophotometer at the exact temperature speci-

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Procedure A: **1-Ethyl-3-(N,N-dimethylcarboxamido)piperidine Hydrobromide** (III).—N,N-Dimethylnicotinamide (25 Gm., 0.167 mole) (IV) and ethyl bromide (48.3 Gm., 0.443 mole) were dissolved in 200–300 ml. anhydrous benzene and refluxed for 48–60 hours. The precipitate formed in the reaction was separated and was dissolved in 150–200 ml. distilled water. The aqueous solution was washed with benzene, treated with charcoal, and filtered. The filtrate was subjected to hydrogenation (aqueous or hydroalcoholic solution) in the presence of 1.0 Gm. platinum oxide (Adams' catalyst) at maximum pressures of 45–50 p.s.i. After absorption of hydrogen ceased, the platinum oxide was filtered off and the solvent was removed by azeotropic distillation under reduced pressure with absolute ethanol and anhydrous benzene. The product was purified by recrystallization.

Procedure B: **1,2-Bis[3-(N,N-dimethylcarboxamido)piperidino]ethane Dihydrobromide** (VI).—N,N-Dimethylnicotinamide (52.1 Gm., 0.347 mole) (IV) and 1,2-dibromoethane (32.6 Gm., 0.174 mole) were dissolved in 200 ml. anhydrous benzene and refluxed for 53–62 hours. The precipitate formed during the reaction was then treated as described under *Procedure A*.

Procedure C: **1-Ethyl-3-(N,N-diethylcarboxamido)-1,2,5,6-tetrahydropyridine Hydrochloride** (XVIII).—1 - Ethyl - 3 - (N,N - diethylcarboxamido)pyridinium bromide (XVII) (2) was prepared by refluxing N,N-diethylnicotinamide (40 Gm., 0.224 mole) (XVI) and ethyl bromide (61 Gm., 0.560 mole) in 250 ml. anhydrous benzene for 48 hours. Excess alkyl halide and solvent were removed by decantation. The residual oil was dissolved in 150 ml. water, the aqueous solution was washed with benzene, and the water was removed under reduced pressure. Last traces of moisture were removed by azeotropic distillation with benzene. The oily product (60 Gm., 0.209 mole) was dissolved in 400 ml. dry methanol and cooled in an ice bath. Sodium borohydride (31.7 Gm., 0.838 mole) was added with stirring to the cold solution over 30 minutes; subsequently the solution was stirred an additional 30 minutes. Methanol was removed by distillation under reduced pressure to give a spongy yellow residue which was dissolved in 230 ml. water and saturated with potassium carbonate. The mixture was extracted with ether, the combined ether extracts were dried over anhydrous potassium carbonate, filtered, and the ether was removed by distillation. The oily residue was purified by conversion to the hydrochloride (anhydrous HCl in anhydrous ether) with or without prior fractionation under reduced pressure. Residual traces of moisture were removed from the oily hydrochloride by azeotropic distillation with benzene. The salt was then purified by recrystallization from ethanol-ethyl acetate.

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Sensitive and Reproducible Assay Method for Chymotrypsin

By B. L. KABACOFF, M. UMHEY, A. WOHLMAN, and S. AVAKIAN

A new assay method for chymotrypsin was developed. This method, based on N-acetyl-L-tyrosine ethyl ester (ATEE), was found to be ten times as sensitive as the proposed N.F. assay method for chymotrypsin. In the new method described, the residual substrate is measured by a single colorimetric determination. The method is convenient and highly reproducible.

THE CLASSICAL assay methods for proteolytic activity such as hemoglobin digestion, casein digestion, and milk clotting, although convenient and simple, lack specificity. N-acetyl-L-tyrosine ethyl ester (ATEE) is a specific substrate for chymotrypsin. The proposed N.F. method for the analysis of chymotrypsin, based on the work of Schwert and Takenaka (1), utilizes ATEE as the enzyme substrate. The rate of hydrolysis of this substrate in pH 7.0 buffer is followed spectrophotometrically at 237 mμ. Readings are taken every 30 seconds for a period of 4 minutes. The temperature must be kept within ±0.1° of 25.0°.

The above method has certain drawbacks which limit its practical application. Elaborate equipment is required to maintain the cell compartment of the spectrophotometer at the exact temperature speci-

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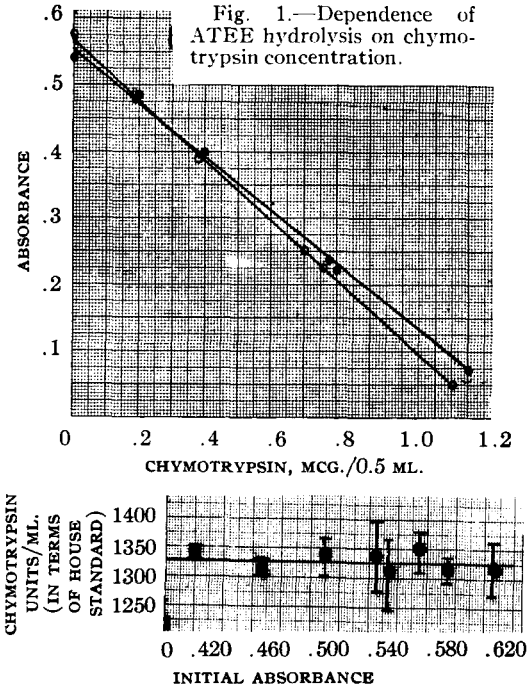

Fig. 1.—Dependence of ATEE hydrolysis on chymotrypsin concentration.

Fig. 2.—Effect of variation of initial ATEE concentration on the assay.

fied. The manipulations involved make readings at intervals of exactly 30 seconds difficult. Variable results may often be obtained when ATEE from different sources is used. Even when the variables are carefully controlled, results are often erratic. The present study was undertaken to devise a new assay method for chymotrypsin based on ATEE and to evaluate its reproducibility and convenience. In this method, the residual ATEE after incubation with the enzyme is measured by reacting it with alkaline hydroxylamine, thus forming the hydroxamic acid (2). The hydroxamic acid is then acidified and reacted with ferric ion to produce a color which is read on a spectrophotometer.

EXPERIMENTAL

The chymotrypsin used in the study was obtained from Princeton Laboratories, Princeton, N. J., and the Worthington Biochemical Corporation, Freehold, N. J. The enzymes from both sources were crystallized. The ATEE was obtained from Mann Research Laboratories, New York, N. Y., Calbiochem, Los Angeles, Calif., and K & K Laboratories, Jamaica, N. Y. The chymotrypsin used as the standard had an assay of 1290 proposed N.F. units/mg. All of the samples utilized in this study were assayed against this standard.

Reagents.—All solutions were prepared with distilled water and reagent grade materials. Hydroxylamine hydrochloride, 139 Gm./L., and sodium hydroxide, 140 Gm./L., were used. Alkaline hydroxylamine solution is prepared by mixing equal volumes of the above solutions just prior to use. Hydrochloric acid (0.001 *N* and 4.0 *N*), phosphate buffer of 1.09 Gm. anhydrous potassium dihydrogen phosphate, and 2.27 Gm. anhydrous disodium hydrogen phosphate diluted to 100.0 ml. (the pH of this solution should be 7.0 ±0.1) were employed. The substrate solution was 115 mg. of N-acetyl-L-tyrosine ethyl ester in 50.0 ml. of phosphate buffer. Shake well to dissolve and filter through Whatman No. 42 filter paper (prepare fresh daily). Ferric chloride (hexahydrate), 189 Gm./L.

Table I.—Comparison of ATEE from Different Sources

	Chymotrypsin Activity, Proposed N.F. Units/mg.		
	Mann	Calbiochem	K & K
CTD-5	1250 ± 41 (4)[a]	1290 ± 69 (4)[a]	1230 ± 43 (2)[a]
CTD-7	1340 ± 13 (4)[a]	1340 ± 26 (4)[a]	1330 ± 20 (2)[a]

[a] Number of determinations.

Method.—A solution of chymotrypsin sample in 0.001 *N* hydrochloric acid is prepared so that it contains 1.5 to 2.0 proposed N.F. units (1.2 to 1.6 mcg.) of chymotrypsin per ml. A solution using chymotrypsin of known potency (standard) is also prepared.

One-milliliter aliquots of the substrate solution are placed into each of three test tubes immersed in a water bath maintained at 25 ±0.5°. To one tube, 0.5 ml. of the sample solution is added. To the second tube, 0.5 ml. of the standard is added. To the third tube, 0.5 ml. of 0.001 *N* HCl is added. The exact time of each addition is noted. It is advisable to stagger the additions 1 minute apart.

Exactly 50 minutes after the addition of enzyme solution or 0.001 *N* HCl to the test tubes, the reaction is stopped by the addition of 2.0 ml. of alkaline hydroxylamine solution. Two minutes later, 1.0 ml. of 4 *N* hydrochloric acid is added to each tube.

The color is then developed by the addition of 1.0 ml. of ferric chloride solution to each tube. The absorbances of the solutions (unknown, standard, 0.001 *N* HCl) are read against distilled water at 540 mμ in a 1-cm. cell. These readings should be made within 20 minutes after the ferric chloride addition. The absorbances of the unknown and standard solutions are each subtracted from that of the 0.001 *N* hydrochloric acid. These differences in absorbance are proportional to the activities of the enzyme solutions. Samples should be run in duplicate or triplicate.

After the addition of each reagent, the tubes should be agitated vigorously. If this precaution is not taken, the absorbance may be erroneously high. The quality of the substrate may be checked prior to the assay by adding all the reagents as stated above using 0.001 *N* hydrochloric acid instead of

Table II.—Variation Within a Single Assay

Sample	Proposed N.F. Units/mg.	Mean	S. E.	Coefficient of Variation %
CTD-5	1320			
	1260	1270	±23	1.8
	1230			
CTD-7	1370			
	1320	1330	±10	0.8
	1310			
6017C	1280			
	1270	1260	±32	2.5
	1220			
6030C	1240			
	1240	1210	±52	4.2
	1150			

TABLE III.—VARIATION AMONG SEVERAL ASSAYS

	Chymotrypsin Activity, Proposed N.F. Units/mg.			
	CTD-5	CTD-7	6017C	6030C
9-21-62	1270	1330	1340	1280
9-24-62	1250	1300	1250	1110
9-25-62	1220	1340	1280	1180
10- 1-62	1290	1350	1240	1190
10- 9-62	1280	1340	1200	1210
10-13-62	1210	1320	1260	1210
Mean	1250	1330	1260	1200
Av. Deviation from Mean	27 (2.2%)	13 (1.0%)	22 (1.7%)	37 (3.1%)

chymotrypsin solution. Do not incubate but read the absorbance immediately. The absorbance should be between 0.400 and 0.650.

RESULTS

Dependence of ATEE Hydrolysis on Chymotrypsin Concentrations.—Figure 1 gives the absorbance obtained after incubation of varying concentrations of chymotrypsin with substrate solution in two typical experiments. Each point on the curves represents duplicate determinations. It can be seen that the relationship is linear up to 1.2 mcg./0.5 ml. Since the absorbance, which is a measure of ATEE present, fell from approximately 0.55 to 0.06, we may say that about 90% of the substrate was hydrolyzed by this concentration of enzyme.

Effect of Variation of Initial ATEE Concentration on the Assay.—Eight solutions of ATEE (from three different suppliers) were made up at various times and were used in assaying lot CTD-7 of chymotrypsin. Although the same amount of ATEE was weighed out, the resulting solution contained variable concentrations of intact ester as evidenced by differences in the absorbances obtained after ferric chloride addition. Because of the slow solubility of ATEE, varying amounts of the ester remained undissolved and were filtered out. Prolonged attempts to effect complete solution of ATEE may result in a variable degree of hydrolysis of the ester.

It can be seen from Fig. 2 that a wide variation of initial ATEE concentration produces no appreciable effect on the assay.

Effect of ATEE from Different Sources on the Assay.—Substrates from three sources were compared using two lots of chymotrypsin (Table I). The greatest variation obtained was that between the Mann and K & K product using CTD-5. This difference was not statistically significant ($P>0.2$).

Variation Within a Single Assay.—Four lots of chymotrypsin were assayed in triplicate. In each case, three aliquots from a single dilution were incubated against the same substrate solution. The results given in Table II are typical. It can be seen that the reproducibility is quite satisfactory.

Variation Among Several Assays.—Four lots of chymotrypsin were assayed in duplicate or triplicate at various times over a period of a few weeks. The results are given in Table III. The average error (deviation from the mean) ranges from 1.0 to 3.1%.

DISCUSSION

In the proposed N.F. assay method for chymotrypsin, the ratio of substrate to enzyme is relatively small (2.5×10^4 on a molar basis). The resulting reaction is frequently not zero order with respect to substrate. As a result, the fall in absorbance is often not linear. In the present study, the ratio of substrate to enzyme is ten times that found with the N.F. method. Therefore, the variable hydrolysis which occurs during the preparation of ATEE solutions is negligible. As was shown in Fig. 2, the initial concentration of ATEE may vary over a wide range without affecting the accuracy of the results. In addition, we found that during the enzymatic reaction, linearity exists even when 90% of the ATEE has been hydrolyzed.

The separate 4-minute incubation required for each sample analyzed by the proposed N.F. method, requires special equipment to preclude temperature variations greater than $\pm 0.1°$. It was found that since with our method, the standard and sample are incubated together for 50 minutes, an ordinary water bath (which may fluctuate within a range of $\pm 0.5°$) was adequate.

In the method under consideration, the absorbance of each sample must be read only once, within 20 minutes after the color development. This is in direct contrast to the repeated 30-second interval readings required with the proposed N.F. method. Additionally, any instability in the spectrophotometer, which is insufficient to interfere with the usual type of assay, will result in a significant error in results obtained by the N.F. method.

The sensitivity of the hydroxamate method as described here is approximately ten times that of the proposed N.F. method. Therefore, any possible interference resulting from other ingredients in chymotrypsin formulations would be greatly minimized by the dilution. At the concentrations utilized in this study, it was found that a 50-minute incubation period provided accurate and reproducible results. When working with lower concentrations, a longer period of incubation may be desirable. Conversely, with larger concentrations, an incubation period of less than 50 minutes may prove satisfactory.

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N-Substituted Cyclopropylamines

By M. FREIFELDER and BRUCE W. HORROM

A NEED IN this laboratory for some *N*-substituted cyclopropylamines of type A prompted an in-

$$\Delta NHCH\overset{\frown}{(CH_2)_n} \quad n = 2 \text{ to } 6$$

A

vestigation of their synthesis. All but dicyclopropylamine were readily obtained by reductive alkylation of cyclopropylamine with the corresponding cyclic ketone in the presence of platinum oxide. Since cyclopropanone was not available, acetone was substituted to give the structurally related *N*-isopropylcyclopropylamine.

Attempts to isolate and distill *N*-cycloheptylidenecyclopropylamine gave a mixture of products of constantly rising boiling points and changing refractive indices. The inability of the product of condensation of amine and ketones to withstand heating in the course of solvent removal discouraged any further effort to isolate and characterize the other intermediate Schiff bases. Each intermediate, however, was identified in solution by means of its infrared spectrum.[1] The amine and ketone (excluding acetone) were mixed in thiophene free benzene. Water separated from the mixture upon standing and anhydrous magnesium sulfate was added. After removal of drying agent, a sample of the solution showed the presence of C=N function by the characteristic band at 6.1 μ. The remainder of the benzene solution was then hydrogenated in the presence of platinum oxide. In the reaction with acetone, equimolar amounts of amine and acetone were mixed, allowed to stand, and then hydrogenated in the presence of platinum oxide.

EXPERIMENTAL

The following is characteristic of the preparation of the *N*-cycloalkylcyclopropylamines listed in Table I.

N-Cyclobutylcyclopropylamine.—A 10-Gm. (0.1428 mole) quantity of cyclobutanone[2] was added to 8.14 Gm. (0.1428 mole) of cyclopropylamine[2] in 50 ml. of anhydrous thiophene free benzene and allowed to stand for 1 hour. Slight warming occurred, and water began to separate. Then 10.0 Gm. of anhydrous magnesium sulfate was added and the mixture allowed to stand for an additional hour. The drying agent was filtered and washed with 25–50 ml. of thiophene free benzene. The filtrate was hydrogenated under 2 atm. pressure in the presence of 0.3 Gm. of platinum oxide. When the uptake of hydrogen was complete (1.5 hours), the benzene solution was filtered from the catalyst. The solvent was distilled off and the residue fractionated. The benzene distillate contained a considerable amount of I. Additional yield as hydrochloride salt was obtained by treating the distillate with alcoholic hydrogen chloride, concentrating the solution to dryness, and recrystallizing the salt from hot acetone.

TABLE I.— *N*-CYCLOALKYLCYCLOPROPYL AMINES PREPARED

ΔNHR

	R	Yield, %	Constants, B.p., °C.	mm.	$n^{25}D$	Hydrochloride m.p., °C.	Formula	Analyses[b,c] C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
I	Cyclobutyl	[a]	130	753	1.4509	...	$C_7H_{13}N$	75.63	74.42	11.78	11.94	12.59	12.48
II	Cyclopentyl	84.3	90–94	68	1.4621	158–160	$C_7H_{14}ClN$	56.93	57.06	9.55	9.57	9.48	9.64
							$C_8H_{15}N$	76.73	77.04	12.08	11.83	11.19	11.09
III	Cyclohexyl	94.1	114–118	34–35	1.4774	169–171	$C_8H_{16}ClN$	59.42	59.54	9.97	9.95	8.66	8.70
							$C_9H_{17}N$	77.63	77.64	12.31	12.51	10.07	10.06
IV	Cycloheptyl	78.4	100–104	40	1.4683	147	$C_9H_{18}ClN$	61.52	61.45	10.32	10.43	7.97	7.99
							$C_{10}H_{19}N$	...	...	...	...	...	...
V	Isopropyl	64.6	98–100	755	1.4136	153–154	$C_{10}H_{20}ClN$	63.30	63.17	10.62	10.94	7.38	7.35
		63.0	...	...	...	163–165	$C_6H_{13}N$	...	...	...	...	...	...
							$C_6H_{14}ClN$	53.12	52.97	10.40	10.66	10.33	10.13

[a] The yield consisted of 46% of material as base and 30% as hydrochloride salt. [b] Rapid absorption of carbon dioxide by the bases made satisfactory analytical values for carbon and hydrogen difficult to obtain. In some instances when the analyses were carried out immediately after distillation good values resulted. In others it was necessary to characterize the compound by means of its hydrochloride salt. [c] Microanalyses carried out by Mr. O. Kolsto and his group in this laboratory.

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[1] Work done in this laboratory by Mr. A. Kammer and Mr. W. Washburn.

[2] The cyclic ketones and cyclopropylamine are available from the Aldrich Chemical Co., Milwaukee, Wis.

In the preparation of the higher homologs, a slight excess of cyclopropylamine was used. Distillation of the resultant products of reduction was uncomplicated.

N-Isopropylcyclopropylamine.—Cyclopropylamine (28.5 Gm., 0.5 mole) and 31.8 Gm. (0.5 mole) of acetone were mixed and allowed to stand for 1 hour, then hydrogenated under 2–3 atm. pressure in the presence of 1.0 Gm. of platinum oxide. After uptake of hydrogen was complete (6 hours), the mixture was allowed to stand until the catalyst settled. The solution was decanted from the catalyst in a nitrogen atmosphere. *Caution!* In air the catalyst may ignite the vapors of the low boiling liquid. The base, which contained some water, was treated with solid potassium hydroxide. It was separated and distilled. A hydrochloride salt was prepared for identification.

In another experiment the reduction was carried out in alcohol. The filtered solution was treated with an equivalent of alcoholic hydrogen chloride and the salt obtained after concentration.

Synthesis of the Diuretic 8-Chloroalloxazine-5,10-dioxide

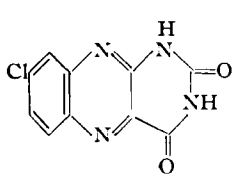
By H. G. PETERING and G. J. VAN GIESSEN

The synthesis of 8-chloroalloxazine-5,10-dioxide, a new diuretic, is described. It has advantages over 8-chloroalloxazine in that it is readily solubilized in aqueous media containing arginine or tris-buffer (THAM).

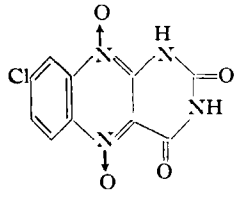
RECENTLY Petering and Van Giessen (1) reported the synthesis of a new diuretic, 8-chloroalloxazine (I) and of several related alloxazines and quinoxalines. In extending the study of the relationship of diuretic activity to chemical structure in this series of compounds, 8-chloroalloxazine-5,10-dioxide (II) has now been prepared. It was found by B. E. Graham in this laboratory (2) to be the only one with diuretic activity comparable to I. Moreover, II can be readily solubilized in water by the addition of arginine or tris-hydroxymethylaminomethane (tris or THAM). Because of the biological potentialities of this compound, its synthesis is presented here.

EXPERIMENTAL

Synthesis.—8-Chloroalloxazine (2.5 Gm.) was suspended in a mixture of 100 ml. 88% formic acid and 10 ml. 30% hydrogen peroxide. The mixture was warmed to 65° on a water bath. The heat was removed as the exothermic reaction began. The reaction temperature was allowed to rise to 95°. (An ice bath was kept on hand to permit rapid cooling in case the reaction became violent.) A rapid evolution of gas occurred when the temperature reached 75–80°, all of the insoluble material went into solution, and the color deepened from pale yellow to orange. Shortly thereafter, crystals of II began to form. After the evolution of gas had ceased, the mixture was allowed to cool to room temperature and was refrigerated at 5° for 48 hours.



8-Chloroalloxazine (I)



8-Chloroalloxazine-5,10-dioxide (II)

The orange crystals were collected and washed with small amounts of cold water, ethanol, and acetone. The yield of air-dried product was 2.4 Gm. or 85% of theory, m.p. 285–292°.

When glacial acetic acid was used as the solvent, II was obtained in 81% yield, m.p. 293–295°.

Recrystallization of II from methylcellosolve raised the melting point to 297–298° (Product A). Recrystallization from a mixture of 88% formic acid containing 10% of hydrogen peroxide (30%) gave 80% yield of II, m.p. 300–301° (Product B). Recrystallization can also be effected from 95% ethanol.

Anal.—Calcd. for $C_{10}H_5ClN_4O_4$: C, 42.8; H, 1.8; Cl, 12.6; N, 20.0; O, 22.8. Found: Product A—C, 43.4; H, 2.4; Cl, 11.7; N, 19.7; O, 23.8. Product B—C, 43.6; H, 1.2; Cl, 12.0; N, 19.2; O, 20.9.

Infrared Spectrum.—The I.R. spectral analyses of Products A and B mentioned above were identical and showed the required bands for the structure proposed for II. N—H, 3140 (sh) and 3050 $cm.^{-1}$; C = 0, 1715–1708 $cm.^{-1}$; C = C/C N, 1608, 1580–1572, 1500(sh), 1480(sh) $cm.^{-1}$; N→0, 1340, 1246, and 1235 (sh) $cm.^{-1}$.

Ultraviolet Spectrum.—The U.V. spectral analyses showed no evidence of the presence of I, but distinctive bands characteristic of II. The ϵ values are as follows: 281.5 mμ, 62,600; 346 mμ, 6150; 459 mμ, 9100.

Chromatography on circular disk paper using as solvent *n*-butanol 6, pyridine 4, water 3 (v/v/v), showed one ultraviolet fluorescing band at R_f 0.48, with a very faint trace of fluorescence at 0.66 which was not identified. The R_f of I in this solvent is 0.81.

8-Chloroalloxazine-5,10-dioxide is soluble to the extent of 12.5 mg. per 100 ml. of water and 6 mg. per 100 ml. of physiological saline. This solubility is raised to 830 mg. per 100 ml. of 0.1 *M* arginine and to 625 mg. per 100 ml. of 0.1 *M* tris-buffer (THAM), with resulting pH values of 7.0 in each case. The dioxide is also solubilized by sodium and potassium bicarbonate.

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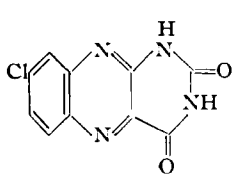
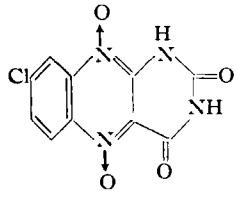
EXPERIMENTAL

Synthesis.—8-Chloroalloxazine (2.5 Gm.) was suspended in a mixture of 100 ml. 88% formic acid and 10 ml. 30% hydrogen peroxide. The mixture was warmed to 65° on a water bath. The heat was removed as the exothermic reaction began. The reaction temperature was allowed to rise to 95°. (An ice bath was kept on hand to permit rapid cooling in case the reaction became violent.) A rapid evolution of gas occurred when the temperature reached 75–80°, all of the insoluble material went into solution, and the color deepened from pale yellow to orange. Shortly thereafter, crystals of II began to form. After the evolution of gas had ceased, the mixture was allowed to cool to room temperature and was refrigerated at 5° for 48 hours.



8-Chloroalloxazine (I)



8-Chloroalloxazine-5,10-dioxide (II)

The orange crystals were collected and washed with small amounts of cold water, ethanol, and acetone. The yield of air-dried product was 2.4 Gm. or 85% of theory, m.p. 285–292°.

When glacial acetic acid was used as the solvent, II was obtained in 81% yield, m.p. 293–295°.

Recrystallization of II from methylcellosolve raised the melting point to 297–298° (Product A). Recrystallization from a mixture of 88% formic acid containing 10% of hydrogen peroxide (30%) gave 80% yield of II, m.p. 300–301° (Product B). Recrystallization can also be effected from 95% ethanol.

Anal.—Calcd. for $C_{10}H_5ClN_4O_4$: C, 42.8; H, 1.8; Cl, 12.6; N, 20.0; O, 22.8. Found: Product A—C, 43.4; H, 2.4; Cl, 11.7; N, 19.7; O, 23.8. Product B—C, 43.6; H, 1.2; Cl, 12.0; N, 19.2; O, 20.9.

Infrared Spectrum.—The I.R. spectral analyses of Products A and B mentioned above were identical and showed the required bands for the structure proposed for II. N—H, 3140 (sh) and 3050 $cm.^{-1}$; C = 0, 1715–1708 $cm.^{-1}$; C = C/C N, 1608, 1580–1572, 1500(sh), 1480(sh) $cm.^{-1}$; N→0, 1340, 1246, and 1235 (sh) $cm.^{-1}$.

Ultraviolet Spectrum.—The U.V. spectral analyses showed no evidence of the presence of I, but distinctive bands characteristic of II. The ϵ values are as follows: 281.5 mμ, 62,600; 346 mμ, 6150; 459 mμ, 9100.

Chromatography on circular disk paper using as solvent *n*-butanol 6, pyridine 4, water 3 (v/v/v), showed one ultraviolet fluorescing band at R_f 0.48, with a very faint trace of fluorescence at 0.66 which was not identified. The R_f of I in this solvent is 0.81.

8-Chloroalloxazine-5,10-dioxide is soluble to the extent of 12.5 mg. per 100 ml. of water and 6 mg. per 100 ml. of physiological saline. This solubility is raised to 830 mg. per 100 ml. of 0.1 *M* arginine and to 625 mg. per 100 ml. of 0.1 *M* tris-buffer (THAM), with resulting pH values of 7.0 in each case. The dioxide is also solubilized by sodium and potassium bicarbonate.

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Sensitive Ultraviolet Spectrophotometric Determination of Some Phenethanolamine Drugs

By LESTER CHAFETZ

The ultraviolet determination of phenylpropanolamine as benzaldehyde after periodate oxidation reported (12) was the basis of a study using a modified procedure for the assay of other phenethanolamine derivative drugs in dosage forms. Quantitative recoveries of aryl aldehyde after periodate oxidation of ephedrine, phenylpropanolamine, phenyramidol, and phenylephrine were demonstrated.

THE UTILITY of ephedrine, phenylpropanolamine, phenylephrine, and other compounds which may be considered phenethanolamine derivatives has led to the publication of a large number of methods for their determination in pharmaceutical dosage forms and other environments (1). Among the methods described for the nonphenolic members of the group are titrations of the base function (2), gravimetric determination *via* the hydrochloride salt (3) or tetraphenylboron salt (4), condensation with chromogens (5), dye-complex colorimetry (6), and absorption spectrometry (7). The monophenolic members of the group have also been determined by methods depending on the presence of that group; among these are bromination (8), coupling with a diazonium salt (9) or 4-aminoantipyrine (10), and the Millon reaction (11). Many other assay methods have been reported for this chemical class, attesting both to their importance and the limitations of sensitivity and selectivity which must be reckoned with in solving each analytical problem.

One of the more selective analytical procedures for a phenethanolamine drug was that described by Heimlich and his co-workers (12) in 1961. They determined phenylpropanolamine in urine by oxidizing the drug to benzaldehyde with periodate and measuring the ether extracted aldehyde spectrophotometrically. The possible application of the periodate oxidation procedure to the assay of ephedrine and related drugs in dosage forms led to the modifications and extensions reported here. The availability of a series of phenethanolaminoheterocyclic compounds synthesized in these laboratories (13) afforded an opportunity to test the procedure on analogs of differing base strength. The chemical structures of the compounds are described in Table I.

EXPERIMENTAL

Reagents.—Two per cent sodium metaperiodate, concentrated hydrochloric acid, approximately 0.05 *N* hydrochloric acid, 0.05 *N* sodium hydroxide, hexane (Phillips high purity grade or equivalent), chloroform A.C.S., and saturated sodium bicarbonate were employed.

Equipment.—A Beckman DU spectrophotometer or equivalent instrument equipped for ultraviolet measurements, Bausch and Lomb spectronic 505 or equivalent recording ultraviolet spectrophotometer (optional), and standard volumetric laboratory glassware were used.

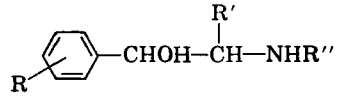
Estimation of Phenethanolamines as Benzaldehyde.—Transfer 5.0 ml. of an approximately 0.12 *M* solution of the nonphenolic phenethanolamine salt to a glass-stoppered tube of about 40-ml. capacity. Add 1.0 ml. of saturated sodium bicarbonate and 0.5 ml. of 2% sodium metaperiodate, and shake 10 minutes. Add 1–2 drops of concentrated hydrochloric acid. (The acid serves to convert amines which are not oxidized by periodate to their nonextractable salts.) Shake the mixture for 30 seconds with exactly 20.0 ml. of hexane, and filter the hexane layer through Whatman No. 1 paper. Determine the absorbance of the hexane extract at 242 mμ *versus* hexane in a 1-cm. silica cell or scan the ultraviolet spectrum in a recording instrument.

Estimation of Phenylephrine as *m*-Hydroxybenzaldehyde.—Transfer 5.0 ml. of an approximately 0.11 *M* solution of phenylephrine hydrochloride to a 60-ml. separator. Add 1.0 ml. of saturated sodium bicarbonate and 0.5 ml. of 2% sodium metaperiodate, and shake mechanically 10 minutes. Add 1–2 drops of concentrated hydrochloric acid. Extract the solution with five 5-ml. volumes of chloroform, and collect the extracts through cotton in a 25.0-ml. volumetric flask. Adjust the solution to the mark, and shake 15.0 ml. with exactly 10.0 ml. of 0.05 *N* sodium hydroxide. Filter the alkali extract through Whatman No. 1 paper, and determine its absorbance at 237 mμ in a 1-cm. cell against 0.05 *N* alkali or scan its spectrum.

RESULTS

Quantitation.—All of the phenethanolamines in Table I gave quantitative amounts of aryl aldehyde, except for the pyrimidine and *s*-triazine derivatives. Unoxidized fenyripol was recovered practically quantitatively and identified by its ultraviolet spectrum. Since the phenethanolamine derivative furnishes an equimolar quantity of aryl aldehyde, a close approximation of the extent of conversion can be made by comparing log molar extinction coefficients of the oxidized phenethanol-

TABLE I.—STRUCTURE OF PHENETHANOLAMINES STUDIED



Compd.	R	R′	R″
Ephedrine	H	CH_3	CH_3
Phenylpropanolamine	H	CH_3	H
Phenylephrine	*m*-OH	H	CH_3
Phenyramidol	H	H	2-Pyridyl
Fenyripol	H	H	2-Pyrimidyl
2-(β-Hydroxyphenethylamino)-*s*-triazine	H	H	2-*s*-Triazinyl

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TABLE II.—SPECTRAL DATA OBTAINED AFTER PERIODATE OXIDATION OF PHENETHANOLAMINES

Compd.	Solvent	λ max.	log ε	$E_{1cm.}^{1\%}$ as HCl salt	
Ephedrine	Hexane	242 mμ	4.15	716.1	
Phenylpropanolamine	Hexane	242	4.14	735.9	
Phenyramidol	Hexane	242	4.15	564	
Benzaldehyde	Hexane	242	4.15	...	(14)
Phenylephrine	Chloroform	257	3.95	441	
		315	3.43	131.8	
	0.05 *N* alkali	237	4.33	1073	
		267*s*	3.84	338	
m-Hydroxybenzaldehyde	Chloroform	...	...	...	
	0.05 *N* alkali	237	4.38		(15)
		267	3.79		
		357	3.41		

TABLE III.—PRECISION OF ABSORPTIVITY VALUES OBTAINED WITH EPHEDRINE HYDROCHLORIDE

Trial	$E_{1cm.}^{1\%}$ value
1	701.7
2	715.3
3	727.3
4	713.0
5	698.3
6	722.2
7	730.7

Mean = 716.1; S.D. = 11.1 units = 1.56%

amines with those of the aryl aldehydes. The absorptivity values ($E_{1cm.}^{1\%}$) of the phenethanolamines as aldehydes are listed in Table II, furnishing another indication of the sensitivity of the analytical method.

To test the feasibility of employing absorptivity values in dosage form assays, the periodate oxidation of ephedrine hydrochloride was replicated over a period of several days. The precision obtained is shown in Table III.

Assays of Ephedrine in Dosage Forms.—The assay procedure was applied to the determination of ephedrine hydrochloride in tablets declaring 1/4 gr. (16.2 mg.) of the drug along with aminophylline, pentobarbital sodium, ethyl aminobenzoate, and aluminum hydroxide, as well as excipients (starch, stearic acid, iron oxide, talc, calcium carbonate, gelatin). A weighed quantity of pulverized tablet representing about 2 mg. of declared ephedrine hydrochloride was shaken with exactly 100.0 ml. of distilled water for 10 minutes in a glass-stoppered 250-ml. conical flask. The mixture was filtered through Whatman No. 1 paper and the first 10–15 ml. filtrate discarded. A 5.0-ml. aliquot was run through the procedure. A scan of the spectrum of the hexane extract showed the characteristic spectrum of benzaldehyde. Using the mean absorptivity value of 716.1, a recovery of 16.5 mg. of ephedrine hydrochloride was obtained, which represents 101.8% of the declared amount. Potassium iodide in a similar formulation interferred with the direct determination of ephedrine by reducing periodate.

Assay of Phenylephrine Hydrochloride in a Dosage Form.—Phenylephrine hydrochloride was assayed in a commercial tablet declaring 2.5 mg. of the drug and 5.0 mg. prophenpyridamine maleate, 194.4 mg. salicylamide, 129.6 mg. of acetophenetidin, 97.2 mg. of aluminum hydroxide, and excipients (starch, talc, magnesium stearate, methyl cellulose, colorant). A quantity of pulverized tablet equivalent to 2 mg. of phenylephrine hydrochloride was shaken mechanically for 10 minutes with exactly 100.0 ml. of 0.05 *N* sulfuric acid and about 25 ml. of chloroform. The acid layer was filtered through paper and two 5.0-ml. aliquots of filtrate were treated as described in the phenylephrine procedure above, omitting addition of periodate for one used as the blank. Using the absorptivity value of 1073, a result of 2.41 mg. of phenylephrine hydrochloride, corresponding to 96.5% of the declared amount, was obtained. A scan of the spectrum showed it to be *m*-hydroxybenzaldehyde.

DISCUSSION

Periodate cleaves carbon-carbon bonds of vic-glycols, α-aminoalcohols where the amine is primary or secondary, and α-hydroxyketones (16). The reaction does not distinguish between *threo* and *erythro* isomers, although the observation that *cis* glycols cleave faster than *trans* (17) with periodate and the use of a difference in cleavage rate of *threo* and *erythro* α-aminoalcohols by lead tetra-acetate (18) suggests that a rate difference might be used as a basis for an analytical method in periodate reactions. The failure of the reaction to proceed with the pyrimidine and *s*-triazine analogs of phenyramidol suggests that the availability of electrons on the exocyclic nitrogen is a factor in the reaction.

Although the data given by Heimlich, *et al.* (12), show a quantitative extraction of benzaldehyde by ether, hexane has advantages in the procedure, since it dissolves compounds having a narrower range of polarity and fewer interferences are possible. Addition of acid before extraction eliminates interference from amine bases. It has been found that iodide causes difficulties, but this and similar interferences might be removed by rational separation techniques (19). The general procedure appears to be applicable with little or no modification to the assays of ephedrine, racephedrine, and phenylpropanolamine and their preparations in the compendia official in the United States.

The sensitivity afforded by the procedure is adequate for its use in dosage form assay. It could be increased by manipulating the volumes employed or by derivatization of the aryl aldehyde formed in a manner analogous to that of McChesney and his co-workers (20).

SUMMARY

Periodate oxidation of phenethanolamine drugs followed by ultraviolet spectrophotometry of the aryl aldehyde formed was shown to be quantitative. It affords a convenient, sensitive, and selective means for the assay of ephedrine in dosage forms,

and it appears applicable to the determination of phenylpropanolamine, phenyramidol, and phenylephrine.

The limitations in selectivity of the periodate procedure and interferences in the method are discussed.

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Molecular Rearrangements, Part I. Edited by PAUL DE MAYO. Interscience Publishers, 605 Third Ave., New York 16, N. Y., 1963. xii + 706 pp. 15 × 23.5 cm. Price $25.

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The Specificity of Serological Reactions. By KARL LANDSTEINER. Dover Publications, Inc., 180 Varick St., New York 14, N. Y., 1962. xviii + 330 pp. 13.5 × 21.5 cm. Price $2. Paperbound.

Reports of studies by Dr. Karl Landsteiner, a 1930 Nobel Prize recipient, on antigens and serological reactions with simple compounds is presented. The work concentrates on the chemical aspects of immunological reactions and provides a concise survey of the basic concepts of immunology. An extensive bibliography of Dr. Landsteiner's writings is appended.

Fatty Acid Metabolism in Microorganisms. By KLAUS HOFMANN. John Wiley & Sons, Inc., 605 Third Ave., New York 16, N. Y., 1963. xii + 78 pp. 12.5 × 19 cm. Price $3.25.

A summarization of recent developments and studies with certain phases of fatty acid metabolism in microorganisms is presented. The volume treats the discovery and chemistry of cyclopropane fatty acids, the chemical nature of monounsaturated fatty acids in bacteria, the quantitative estimation of fatty acids in bacterial lipids, the biosynthesis of the cyclopropane ring, and the anaerobic biosynthesis of monosaturated fatty acids in microorganisms. The necessary experimental work was done in the chemistry and biochemistry departments of the University of Pittsburgh by the author.

British Pharmaceutical Codex 1963. COUNCIL OF THE PHARMACEUTICAL SOCIETY OF GREAT BRITAIN. The Pharmaceutical Press, 17 Bloomsbury Square WC 1, London, England, 1963. xxxvi + 1433 pp. 14 × 22 cm. Price $23.75.

This edition of the *Codex* again presents authoritative and useful therapeutic information as well as recognized and accepted standards for many pharmaceuticals and dosage forms not included in the *British Pharmacopoeia* in the usual high tradition of this compendium. The general format and arrangement found so popular and convenient with prior editions has been retained but the publication schedule has been accelerated to parallel that of the B. P. Several of the monographs incorporate infrared identification procedures; appropriate authentic specimens for the infrared procedures have been established. Over 200 new admissions are included in this edition and, as in the past, Parts II through VI provide monographs on immunological products, human blood preparations, surgical sutures, surgical dressings, and formulary preparations. In addition, several new appendixes have been added covering aerosol propellants, a system for the classification of powders, a table of standard wire gauges, and additional analytical information to aid the examination of various pharmaceutical products. Undoubtedly, pharmaceutical and medical practitioners in the United Kingdom will continue to place heavy reliance upon the *Codex* for the most concise and reliable information on today's leading pharmaceutical products and pharmaceutical scientists in the United States will find this edition a useful reference.

Alternating Current Polarography and Tensammetry. By B. BREYER and H. H. BAUER. Interscience Publishers, 605 Third Ave., New York 16, N. Y., 1963. xix + 288 pp. 15 × 23.5 cm. Price $12.

This thirteenth volume in the "Chemical Analysis" series presents a monograph on what is probably the most active area of electrochemical techniques. The work includes a chapter which provides a historical introduction to a.c. polarography and tensammetry, another giving a connected account of quantitative theoretical treatments of electrode processes under the influence of small superposed alternating voltages, another describing the necessary apparatus in detail. In addition, other chapters discuss the main results which have been obtained in the study of various organic and inorganic substances and mechanisms of electrode processes. While the book would serve best to acquaint the reader with the essentials of this technique, the presentation has been arranged so that relevant information on the analytical uses of the a.c. technique is readily accessible.

Handbook of Ocular Therapeutics and Pharmacology. Edited by PHILIP P. ELLIS and DONN L. SMITH. The C. V. Mosby Co., 3207 Washington Blvd., St. Louis 3, Mo., 1963. 193 pp. 17 × 24.5 cm. Price $8.50.

An encyclopedia-like handbook on ocular therapy and therapeutics is presented in this brief volume. One section covers the therapeutic aspects of the more common ocular diseases in summary form and another discusses the pharmacology of therapeutic agents employed. The pharmacology section discusses aspirin to zinc sulfate as related to ocular therapy quite adequately.

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